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B. All components of abbreviations with initial capitals (not as European usage in World List *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

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E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

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The Reproductive Cycle and Spawning in a Caribbean Gorgonian

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Abstract. The reproductive biology of *Plexuara* A, a common but undescribed Caribbean gorgonian, was studied in the San Blas Islands, Panama. Oocytes were present in the polyps throughout the year, though a seasonal developmental cycle is evident. Early stage oocytes appeared at the base of the polyps in November. In January total egg volume per polyp began to increase and reached a maximum in early May. Total egg volume per polyp then decreased through the summer as mature eggs were released. Spawning occurred over a period of 4–7 days following each full moon in May, June, and July. Egg release was synchronous, starting at approximately 18:30 and lasting 90 minutes. Larval development is initiated just prior to or at the time of release. Initiation of larval development at the time of release without brooding is unknown for any gorgonian or scleractinian. Of 265 colonies examined from 6 reefs near San Blas Point, all but 3 contained gonads and were female. No male or hermaphroditic colonies have been found in the San Blas Point population. The scarcity or possible absence of males suggests that the eggs may develop parthenogenetically. Both of these unusual characteristics point to the need to use caution whenever the reproductive biology of a coral is described without an exhaustive examination of the complete reproductive cycle.

Introduction

Gorgonians are an important component of many Caribbean reefs (Kinzie, 1970, 1973; Goldberg, 1973; Opresko, 1973; Muzik, 1982; Lasker and Coffroth, 1983). In some habitats they comprise the dominant coral cover, yet knowledge concerning the reproductive biology of these organisms is lacking. In contrast, sexual reproduction in scleractinians has become a topic of renewed interest (for review see Fadlallah, 1983; Harrison, 1985). This interest is due in part to the diverse assortment of reproductive strategies employed by scleractinians (Harrison, 1985; Szmant, 1986) and anthozoans in general (Chia, 1976). Compared to other anthozoan taxa, gorgonians exhibit a more limited range of reproductive strategies. Most gorgonians appear to be gonochoric brooders (Kinzie, 1970; Grigg, 1977; Weinberg and Weinberg, 1979; Brazeau, 1986). The combination of separate sexes and internal fertilization (or at best fertilization on the surface of the female colony) is an unusual strategy in other anthozoan taxa and is the rarest strategy displayed by the ecologically similar scleractinians (Szmant, 1986). The wide distribution and apparent success of gorgonians throughout the Caribbean and their reliance on a relatively unusual reproductive strategy suggests the need for more information on the basic reproductive biology of this group.

In this paper we report on reproduction in a Caribbean gorgonian. The species, a *Plexuara* sp. of uncertain affinity, is similar to *Plexuara homomalla* forma *kükenthali* and will be referred to as *Plexuara* A (after Lasker, 1984). *Plexuara* A is common in the San Blas Islands, Panama, and is locally abundant at sites throughout the Caribbean (Lasker, unpub. data). We present data on colony size at first reproduction, the annual cycle of gonad development, mode of reproduction, and timing of egg release.

Materials and Methods

Samples of *Plexuara* A colonies were collected from Korbiski reef, a small back reef and reef flat complex located near the Smithsonian Tropical Research Institute field station in the San Blas Islands, Panama (9° 33′N; 78° 52′W). To determine colony size at first reproduction, all *Plexuara* A colonies within a 10 × 10 m area were

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examined *in situ* in July 1984. All colonies with visible gonadal material were scored positive for reproductive ability. To follow the yearly reproductive cycle, 10 colonies were tagged with aluminum tags nailed to the reef substrate and sampled monthly from July 1983 to July 1985. Multiple sampling of these colonies did not affect the results since there was no systematic change in reproductive state of the colonies over the two-year period. Samples were fixed in 10% formalin and later examined with a binocular stereomicroscope. Twenty polyps located between 5 and 15 cm from the branch tip were arbitrarily selected and the diameters of all oocytes were measured with an eyepiece micrometer. To follow oocyte development up to and during egg release, weekly samples of 5 tagged colonies were collected in May, June, and July of 1986 and 1987. During the spawning period in 1987, samples were taken twice daily from the same 5 colonies. The reproductive cycle of *P. homomalla,* a close congener of *Plexuara* A, was also followed on the same reef to determine if the two reproductive cycles were in synchrony. Samples from 5 tagged *P. homomalla* were collected monthly from July 1983 to July 1985 and weekly during May, June, and July of 1987.

Since none of the collections of *Plexuara* A from 1983, 1984, and 1985 included male colonies, 140 additional colonies were sampled from Korbiski reef in July of 1986 to determine the sex ratio of *Plexuara* A. Twenty colonies each were also collected from Macaroon, Sail Rock, Tiantupo, and T-Bar in 1987 to determine the sex ratio on these nearby reefs. Additionally, to determine if sex varied within colonies, 10 colonies were selected at Korbiski in July 1988. Sections of tissue from the outer, middle, and base of each colony were collected for sex determination.

To determine the sex and gonad developmental state of each sample, cross- and longitudinal-sections were decalcified, dehydrated, cleared, embedded in Paraplast, sectioned 7 μm thick, and stained with Heidenhain's azocarmine-aniline blue (Yevich and Barszcz, 1977).

To observe egg release, a series of night dives were undertaken in May, June, and July of 1987 and 1988. During each dive in 1987, all colonies in 4 areas, approximately 100 m^2 each, were monitored. Colonies that released eggs were tagged each night and counted the following morning. In 1987 and 1988 eggs were collected with a large syringe as they emerged from the polyps. Eggs and larvae were fixed in 10% formalin and later embedded, sectioned, and stained as noted above.

Results

Colony size and gonad development

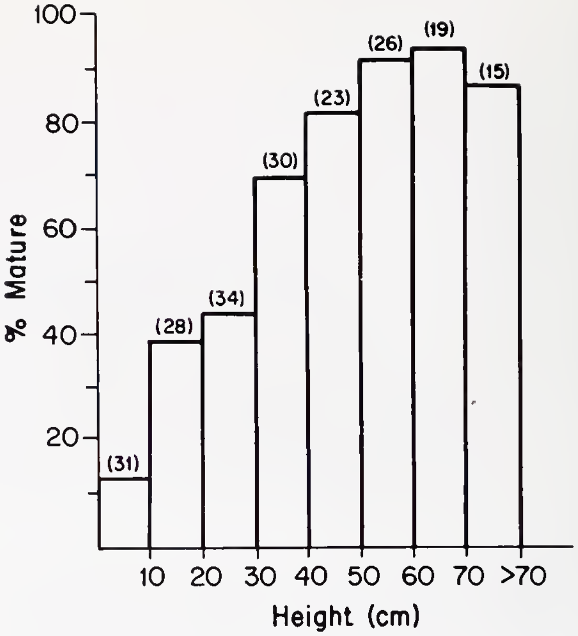

Figure 1. Proportion of *Plexuara* A colonies containing visible gonad. Colonies sampled in 1984 from a 10 × 10 m site at Korbiski reef. Number sampled in each size class is noted in parenthesis.

Figure 1 shows the percentage of colonies in 10 cm increment size classes which contained visible gonads in July 1984. The presence of visible gonads is a good indicator of reproductive activity since only large mature gonads are visible to the naked eye. Of the 206 *Plexuara* A colonies, 58% had visible (mature) gonads. Small colonies, 30 cm or less in height, less frequently had mature gonads (32%), whereas 80% of the colonies greater than 30 cm in height had mature gonads. The smallest colony found to be reproductive was 10 cm in height.

Sexuality

No male or hermaphroditic polyps or colonies were found among the 265 colonies examined over 4 years from 6 reefs in the San Blas Point area. During the reproductive months of May, June, and July, all but three of the colonies examined histologically contained ovaries. There were no colonies in which the gender was ambiguous. From these data we conclude that male colonies are extremely rare or absent in this area.

Annual cycle of oocyte development

Plexuara A displays a yearly or multi-year cycle of oogenesis. Young oocytes appeared in the polyps each November (Fig. 2a). The oocytes were attached to the septa at the base of each polyp by a short mesogloeal stalk. Larger oocytes, presumably from the previous year, were also present in November. The young oocytes increased in size and by January were indistinguishable from the older oocytes. From this pool of oocytes a portion began

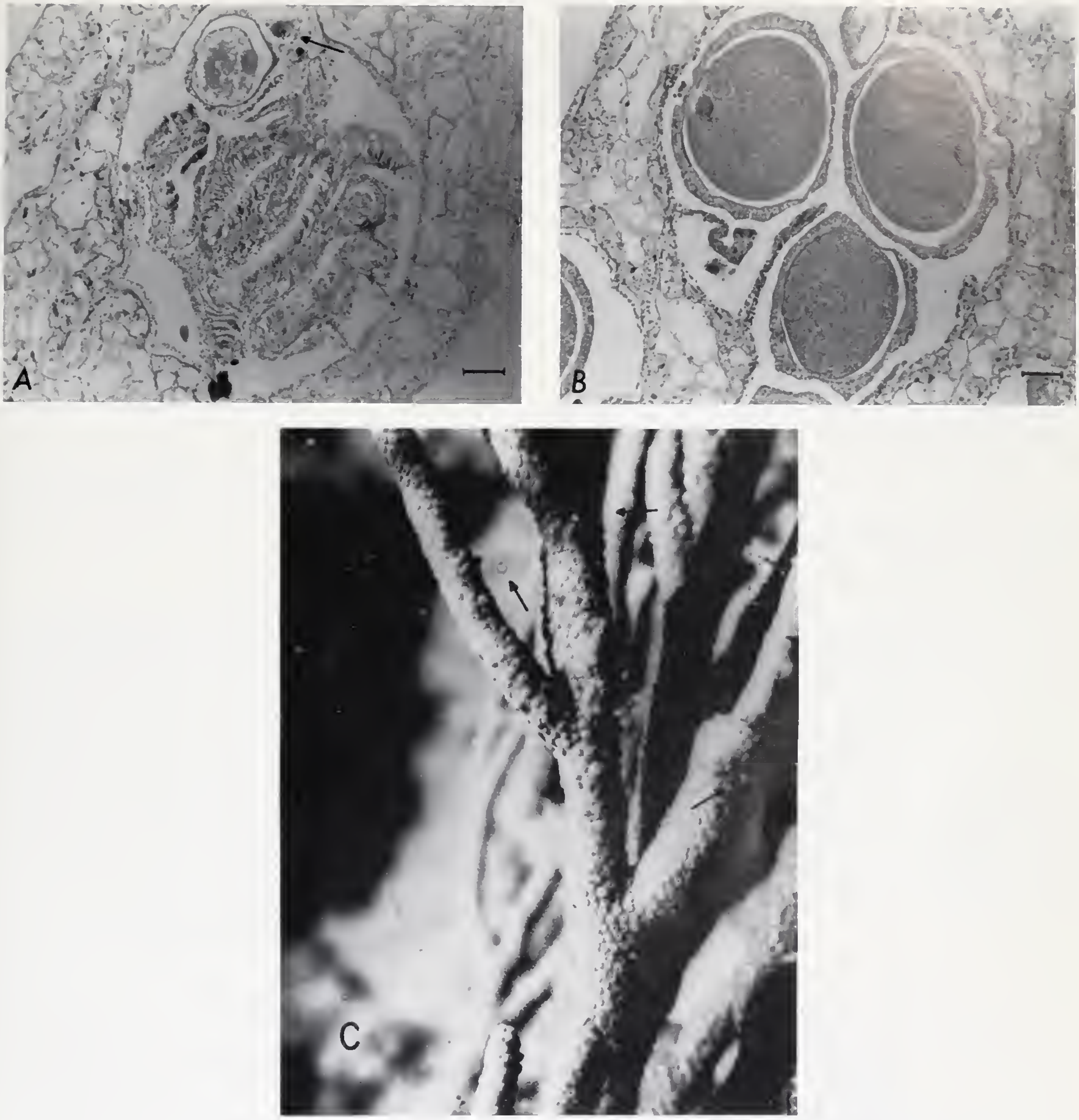

Figure 2. *Plexaura* A. Stages of oogenesis. (A) Young oocyte. Arrow indicates young oocyte. Note large, older oocyte also present. (B) Large oocytes at base of polyp. (C) Eggs at time of release. Arrows indicate eggs just released and eggs emerging from polyps. Scale bar = 125 μm.

to increase in size rapidly. An average of 1.9 oocytes/polyp (SD = 0.99) reached maturity each year. Maximum egg diameters ranged from 500 to 600 μm (Fig. 2b). The cycle of development can be seen in Figure 3, which shows mean total egg volume per polyp from June 1983 to July 1985. Since oocytes were present in the polyps throughout the year, mean egg volume never decreased to zero. Total egg volume per polyp increased slightly in November of each year with the appearance of the new oocytes, and increased rapidly from January through May as a portion of the oocytes increased in size. In June, July, and August, total egg volume decreased as the large

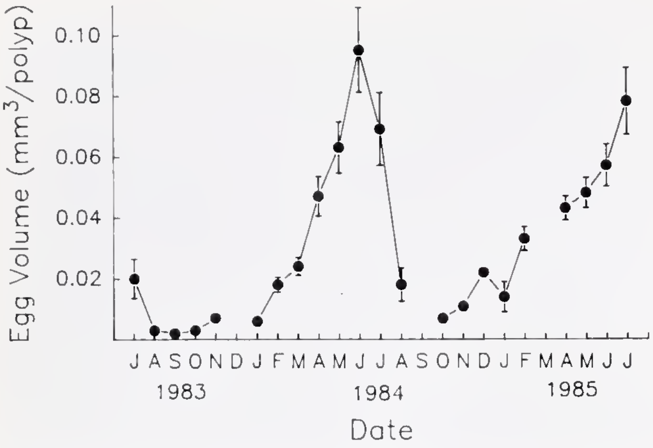

Figure 3. Total egg volume per polyp for *Plexuara* A. Based on means of 20 polyps from each of 10 colonies. Error bars denote 95% confidence intervals. Samples were not collected in Dec. 1983, Sept. 1984, and Mar. 1985.

eggs were released. Large eggs (>400 μm diameter) that were not released degenerated and were not present in September samples.

The seasonal pattern of oocyte development can also be seen in Figure 4, which shows the number of eggs per polyp in three distinct size classes. The number of eggs in the smallest size class (<200 μm diameter), though variable, did not exhibit any seasonal pattern. This size class represents the common pool of oocytes present in the polyps throughout the year. Numbers of eggs in the middle size class (200–400 μm diameter) increased in early spring as eggs enlarged and then decreased in May and June as the oocytes grew into the >400 μm size class. The decrease in the number of eggs in the largest size class in July and August was coincident with spawning.

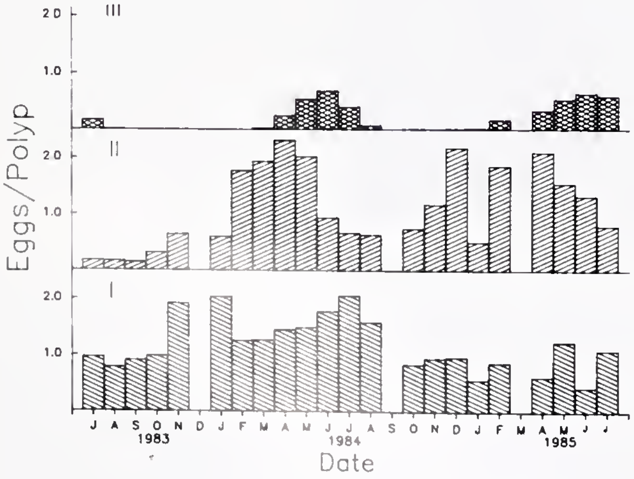

Figure 4. Monthly changes in mean number of eggs per polyp for *Plexuara* A in three size classes. I, <200 μm diameter; II, 200–400 μm diameter; III, >400 μm diameter.

Table I

Numbers of Plexuara *A colonies observed to spawn in 1987 at Korbiski reef*

Month	Site	Colonies spawning	Total number of colonies (>20 cm)	Percent of colonies spawning
May	10 × 10	13	95	13.7
	I	11	84	13.1
	III	6	106	5.7
		30	285	10.5
June	10 × 10	40	95	42.0
	I	37	84	44.1
	II	52	144	36.1
	Wall	35	114	30.7
		164	437	37.5
July	10 × 10	39	95	41.0
	II	64	144	44.4
		103	239	43.1

Spawning

Mature eggs (eggs in which the germinal vesicle had broken down) were released following each full moon during the summer months. In May, June, and July of 1987 and 1988, mature eggs were released over 4–7 day periods, 1–2 days following each full moon (Fig. 2c). Spawning was highly synchronous. Colonies began releasing eggs at virtually the same time shortly after sunset (18:30–18:50). The period of release lasted approximately 90 minutes each night. Histological examination of branch samples collected twice daily during the spawning period did not reveal any developing larvae within the polyps nor any eggs in which the germinal vesicle had broken down. However, eggs collected immediately after release and held in polyethylene containers developed into planulae.

Table I shows the number of colonies that released eggs from four sites at Korbiski in 1987. These numbers are a minimum estimate of spawning colonies since it was not possible to simultaneously monitor all colonies during the periods of egg release. Although some small colonies contained oocytes, we never observed egg release in colonies less than 20 cm in height. The percentage of colonies releasing eggs was greatest in June and July and varied between sites. Many colonies spawned on more than one night in any given month (48% of the colonies in June and 32% in July) and in more than one month. This pattern is also evident in Figure 5, which shows the number of large (>400 μm) eggs per 20 polyps during 1984. The three colonies were chosen to demonstrate the variability in times of maximal development and release among colonies. Colonies had peak egg con-

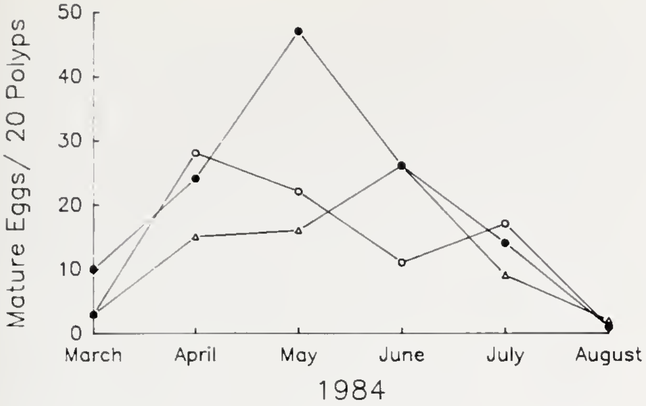

Figure 5. Total number of large eggs (>400 μm) in 20 polyps of three *Plexuara* A colonies. Colonies were chosen to demonstrate the variability in times of maximum development and release.

tent in different months and also differed in which month(s) release occurred.

Discussion

As in other gorgonians, *Plexuara* A colony size was an important determinant of reproductive state. Wahle (1983) reports that only *Plexaura homomalla* colonies that are greater than 20 cm in height are reproductive. Our results are similar to Wahle's in that most colonies less than 20 cm in height were not reproductive. Furthermore, <20 cm tall colonies were never observed to release eggs. This indicates that either the eggs fail to mature or do so in very small numbers in small colonies. Unlike Wahle's observations, we did find reproductive tissue in some <20 cm colonies and even in some 10 cm colonies. The presence of gametogenesis among these colonies may indicate a smaller size of first reproduction among *Plexuara* A colonies, error introduced in using colony height to measure size of first reproduction, or effects of the age of the colony. First, *Plexuara* A may start reproduction at a smaller size than *P. homomalla.* Size of first reproduction varies among colonial coelenterates such as alcyonaceans (Benayahu and Loya, 1984) and scleractinians (Szmant, 1986). It is likely that gorgonians also will exhibit interspecific variation in this basic life history trait. Second, and perhaps most important is the fact that colony height is an approximate measure of overall colony size for organisms with arborescent growth forms like *Plexuara A.* Colonies of similar height often vary in the number of branches and thus in total biomass. More precise estimates of size may identify a less variable size of first reproduction. Finally, *Plexuara* A colonies of the same size vary in age. Growth of colonies monitored in the field in the San Blas indicate that 5 years may be the minimum time required for a colony to reach 20 cm in height (Lasker, unpub. data), but the commonness of severe grazing among small colonies suggest that it may take many colonies substantially longer to reach 20 cm. Furthermore, many *Plexuara* A colonies develop from fragments broken off of large colonies (Lasker, 1984). Thus a small colony could come from a clone that is tens or hundreds of years old. Although we consider it unlikely that age alone has a significant effect on reproductive state, Hughes and Connell (1987) observed an age effect in the growth and mortality rates of some Great Barrier Reef scleractinians.

The mean diameter of mature oocytes in *Plexuara* A is large compared to many scleractinians, but similar to the large eggs seen in other octocorals (Benayahu and Loya, 1986). In addition, the number of mature eggs per polyp is low compared to most anthozoans, but similar to the values reported for other gorgonians (Table II). Alcyonaceans, like gorgonians, also produce large eggs but differ in that they produce many more eggs per polyp. The basis for the difference between the two alcyonarian orders is unknown but may be a simple function of the length of polyps.

One aspect of the reproductive biology of *Plexuara* A that differs from that of gorgonians and other corals is the pattern of oocyte development. Oocytes are present in *Plexuara* A colonies throughout the year. New oocytes are added to this standing pool of oocytes each November, and in January a portion of the oocytes initiate further development into large mature eggs. Large eggs that are not released degenerate and are resorbed by the polyps, however the small oocytes appear to persist through the reproductive season. This cycle suggests oocyte maturation time requires more than a single season. Two-year maturation times have been reported for the alcyonaceans, *Lobophytum crassum* (Yamazato *et al.,* 1981)

Table II

Mode of reproduction and number of mature eggs/polyp for gorgonian species

Gorgonian species	Mode of reproduction	Mature eggs/polyp	Reference
Briareum asbestinum	External Brooder	1.8	Kinzie, 1970; Brazeau, 1986
Eunicella singularis	Brooder	7	Weinberg and Weinberg, 1979
Eunicella stricta	Brooder	4	Theodor, 1967
Muricea californica	Brooder	1.6	Grigg, 1977
Muricea fruticosa	Brooder	3.8	Grigg, 1977
Plexaura A	Broadcast	1.9	
Pseudopterogoria elisabethae	Unknown	7	Kinzie, 1970
Pseudopterogoria bipinnata	Broadcast	7	Kinzie, 1970

and *Sarcophytum glaucum* (Benayahu and Loya, 1986) and the gorgonian *Corallium rubrum* (Vighi, 1972, in Yamazato *et al.*, 1981). However, two discrete size classes of oocytes could be identified in the polyps of these species. In *Plexuara* A, the primary oocytes that appear in November increase in size, and become indistinguishable from the older oocytes already present. Thus, the actual time for oocyte maturation is, at minimum, 7 to 9 months, but may be longer. To our knowledge, the maintenance of a pool of oocytes throughout the year is unknown for any coral. It may represent a mechanism by which mature egg production can be adjusted later in the developmental season (Jan.), rather than at the time of primary oocyte appearance (Nov.).

Spawning by *Plexuara* A colonies is extremely predictable. *Plexuara* A colonies release eggs at virtually the same time over 4 to 7 nights following the full moons of May, June, and July. The duration of egg release each night is also fairly constant (approximately 90 min). In addition, two other gorgonians release planulae and/or eggs on some of the same nights, *Briareum asbestinum* (planulae) and *Pseudoplexaura porosa* (eggs?) (pers. obs.). Although seven species of soft corals participate in the mass spawning events on the Great Barrier Reef (Babcock *et al.*, 1986), this is the first reported incidence of synchronous spawning in Caribbean gorgonians. A number of Caribbean scleractinians also spawn following summer full moons (Szmant, 1986), but it is not known whether these species spawn at the same time as *Plexuara* A. Like many of the Great Barrier Reef species, the time at which *Plexuara* A colonies began to release eggs each evening was fairly precise (18:34, ±10 min.). The net result is one of a large number of eggs being released during the short period of time after sunset, but before moonrise. Such a strategy would be expected if visual predators are an important cause of larval mortality. Lasker (1985) reported that at least one predator, the butterflyfish *Chaetodon capistratus*, showed increased feeding activity on *Plexuara* A polyps on the days prior to egg release, and we observed *C. capistratus* feeding on released eggs immediately after sunset, at the start of spawning.

One of the most surprising aspects of *Plexuara* A reproduction in the study population was development of larvae from eggs released by the female colonies despite the extreme rarity or absence of males. In many gorgonians, spermaries develop rapidly within weeks prior to spawning. Among these species, samples taken at times other than during the peak reproductive period exhibit either skewed sex ratios or the total absence of males (Goldberg and Hamilton, 1974; pers. obs.). However, this does not explain our failure to locate male colonies in the San Blas Point population. Samples collected for sex determination were always collected during the reproductive period and all colonies were positively identified as female. Histological examination of male colonies of the close congener, *P. homomalla*, indicate that this species does not spawn at the same time and therefore cannot fertilize the eggs of *Plexuara* A. Histological examination of polyps before and after spawning show that the larvae arise from eggs (*i.e.*, are not produced vegetatively, independent of the gametes). Thus, the production of larvae by *Plexuara* A has two possible explanations. The first explanation is that fertilization has occurred despite an enormously skewed sex ratio. If this is the case then the extremely rare males must enjoy high fitness. The second explanation is that the eggs develop parthenogenetically. Parthenogenesis has been reported for only one octocoral, *Alcyonium hibernicum* (Hartnoll, 1977) and is unknown in scleractinians. Asexual planulae have been reported in *Pocillopora damicornis* (Stoddart, 1983) and *Tubastrea diaphana* and *T. coccinea* (Ayre and Resing, 1986) but in all three the brooded asexual planulae appear to be derived independent of the gonads (Stoddart and Black, 1985; Ayre and Resing, 1986). The possibility of parthenogenesis is especially interesting given that *Plexuara A* also propagates via fragmentation (Lasker, 1984). Thus, *Plexuara A* clones may spread between reefs via the dispersal of parthenogenetic eggs as well as locally via fragmentation. Consequently, clones may exhibit wide geographic distributions.

Plexuara A also exhibits a unique developmental pattern among anthozoans. Eggs initiate development almost simultaneously with release from the polyps (with or without fertilization). The possibility that development is parthenogenetic confounds the interpretation of the reproductive strategy, but regardless of the genetics, the strategy is enigmatic. Like all octocorals examined to date, *Plexuara* A produces large eggs (Benayahu and Loya, 1986). However, most octocoral species, and all gorgonians, brood their eggs either within the polyp (Kinzie, 1970; Hartnoll, 1977; Weinberg and Weinberg, 1979; Sebens, 1983), or on the colony surface (Benayahu and Loya, 1983; Brazeau, 1986; Farrant, 1986). The production of a large and presumably costly egg, which is then cast into the water column, runs counter to the hypothesis that brooding has evolved as a mechanism of avoiding larval predation. Similarly, the initiation of development and the slow development of the embryos are unlike the known examples of broadcast spawners, where outcrossing, dispersal, and escape from water column predators are the presumptive selective agents. *Plexuara* A, like most octocorals, does not readily fit into reproductive strategies designed to explain scleractinian patterns. It is likely these models will require the inclusion of taxonomic/morphologic constraints and/or additional ecologic parameters in order to explain the reproductive patterns seen in octocorals.

Finally, it should be noted that from the examination of our monthly samples one might conclude that *Plexaura* A is a typical broadcast spawner. Only the observation of the actual reproductive event showed this assumption to be incorrect. As was first demonstrated by the discovery of the Great Barrier Reef mass spawning event (Harrison *et al.*, 1984; Babcock *et al.*, 1986), detailed field observations of reproductive events are a necessary component of discerning reproductive strategies among anthozoans. Detailed examinations of anthozoan life cycles have found exceptions to the traditional concepts of reproductive strategies (Stoddart, 1983; Ayre and Resing, 1986) and further work will undoubtedly discover additional exceptions.

Acknowledgments

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Characterization of a Factor with Oocyte Maturation Inducing Activity in *Spisula*

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Abstract. A substance that induces germinal vesicle breakdown (GVBD) of *Spisula* oocytes *in vitro* was isolated from *Spisula* body fluid (SBF) and ganglion cells by ultrafiltration through PM-10 membrane filter, charcoal extraction, and reversed phase HPLC. Its retention time on HPLC elution is coincident with that of reference serotonin (5-hydroxytryptamine, 5-HT) creatinine sulfate. The HPLC-purified substance from SBF and nerve ganglion induced GVBD in *Spisula* oocytes in a dose-dependent manner. 5-HT was identified in *Spisula* body fluid and ganglion cells by HPLC-electrochemical detection. The present results show that the factor in *Spisula* body fluid and ganglion cells that induces GVBD in *Spisula* oocytes *in vitro* is 5-HT or a closely related compound.

Introduction

Full-grown, naturally shed *Spisula* oocytes are arrested at the dictyate stage of meiosis and retain intact germinal vesicles (Allen, 1953). Upon fertilization meiosis is resumed, leading to germinal vesicle breakdown (GVBD). Oocyte maturation can be induced by chemical agents including KCl and ionophore A23187 (Allen, 1953; Schuetz, 1975).

Serotonin (5-hydroxytryptamine, 5-HT), a neurotransmitter, induces spawning in marine bivalves (Matsutani and Nomura, 1982). Many species of bivalves contain 5-HT in the nervous system (Welsh and Moorhead, 1960; Malanga *et al.*, 1972; Hiripi and Osborne, 1976; Stefano and Catapane, 1977; Smith, 1982). 5-HT has been detected in the central nervous system and gonads of the scallop, *Patinopecten yessoensis* (Matsutani and Nomura, 1984). Hirai *et al.* (1988) demonstrated that 5-HT injected into *Spisula* gonads induced spawning. When 5-HT is added to the suspending medium of *Spisula* oocytes, GVBD is induced. Recently, Toraya *et al.* (1987) reported that *Spisula* body fluid contains a substance that potentiates 5-HT action in inducing oocyte maturation.

The present study was undertaken to purify and characterize the substance with oocyte maturation inducing (OMI) activity from *Spisula* body fluid and ganglion cells. Evidence shows that the OMI activity in *Spisula* body fluid and nerve ganglion cells is structurally related to 5-HT.

Materials and Methods

Chemicals

5-HT creatinine sulfate and trypsin were purchased from Sigma Co. (St. Louis, Missouri); Norit A (activated charcoal) from Fisher Chemical Co. USA; methanol, acetone, acetic acid, and hydrochloric acid (all HPLC grade) from T. J. Baker Chemical Co. (New Jersey). Artificial seawater (ASW) (Cavanaugh, 1974) was obtained from the Chemical Room, Marine Biological Laboratory (Woods Hole, Massachusetts).

Preparation of oocytes

Individuals of *Spisula solidissima* were kept in a laboratory aquarium with cold running seawater at the Marine Biological Laboratory (Woods Hole, MA) until used in the study. Ovaries from *Spisula* females were excised, minced in ASW, and strained through a pad of cheesecloth into a large beaker containing ASW. The oocytes were settled by gravity and the supernatant aspirated off. The washing procedure was repeated at least three times. The washed oocytes were used in the assay.

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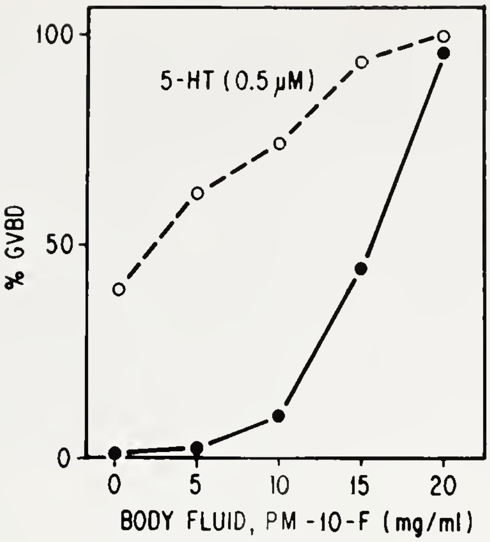

Figure 1. Dose-dependent GVBD-inducing activity of *Spisula* body fluid. The ultrafiltrate (SBF-PM-10F) was tested alone (● —— ●) and with serotonin (○ --- ○). Assay was performed as described under Materials and Methods.

Assay for maturation inducing activity

Samples to be assayed for OMI activity were dissolved in 1.0 ml of ASW and placed in a Falcon tissue culture dish (35 mm). One drop of *Spisula* oocytes (about 2000) was added and incubated at room temperature. After 30–40 min, oocytes were examined under a light microscope to determine the presence or absence of germinal vesicles. More than 100 oocytes were scored for each determination. The percentage of oocytes showing GVBD was calculated from the total number of oocytes scored. The values shown in the figures are the mean of three different experiments.

Purification of the factor with OMI activity

Spisula body fluid (SBF): body fluid was obtained by cutting the adductor muscles and the mantle and collecting the mantle fluid. The pooled body fluid was filtered through cheesecloth and centrifuged at 3000 × *g* for 20 min. The supernatant fluid was lyophilized, assayed for OMI activity, and purified.

OMI activity was purified by the following steps:

Step I. The lyophilized SBF was resuspended in distilled water (half the original volume) and centrifuged. The supernatant (200 ml) was filtered through a PM-10 membrane (Amicon Corp.) fixed in a 200 ml Amicon pressure cell under N_2 pressure. The filtration was performed in a cold room for approximately 24 h. The filtrate (175 ml) was placed in dialysis tubing with mol. wt. cut off of 1000 daltons and dialyzed against distilled water. The PM-10 filtrate (PM-10-F) and the retentate (DR) were assayed for OMI activity using *Spisula* oocytes.

Step II. Activated charcoal (30 mg/ml) was added to the PM-10-F sample and the mixture stirred slowly at 4°C for 30 min. The mixture was centrifuged at 800 × *g* for 10 min at 4°C. The sediment was resuspended in an equal volume of 0.1 *N* HCl, stirred for 30 min, and centrifuged at 3000 × *g* for 20 min at 4°C. The supernatant was filtered through a Whatman No. 1 filter paper. The filtrate was lyophilized and further purified on HPLC.

Step III. The test samples (100 μg/200 μl) were injected into a reverse-phase partisil-10 ODS-3 column (4.6 × 250 mm) (Whatman Inc., Clifton, New Jersey). A short precolumn (5 × 60 mm) packed with an octadecyl-coated pellicular support (Co. Pell ODS, Whatman) was used as the guard column. The column was eluted in isocratic run with 0.1 *M* acetic acid at a flow rate of 1 ml/min. Each pooled fraction was lyophilized and assayed for the OMI activity using *Spisula* oocytes.

High-performance liquid chromatography with electrochemical detection (HPLC-ECD)

Spisula body fluid (SBF) extract was prepared as described above. The nerve cell extract (SME) was prepared by the method of Smith (1982). The sample extract

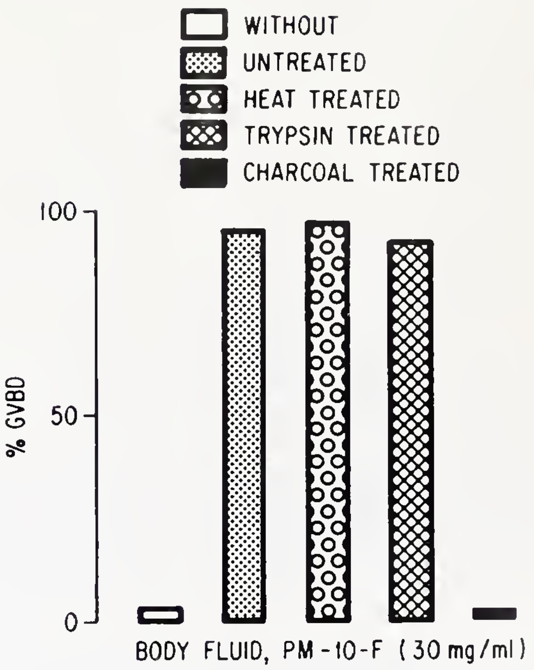

Figure 2. GVBD inducing activity of *Spisula* body fluid after treatment by heating, with trypsin and charcoal extracted. Fraction SBF-PM-10F was used. The procedures for the heat and trypsin treatments, charcoal extraction, and the bioassay are described under Materials and Methods.

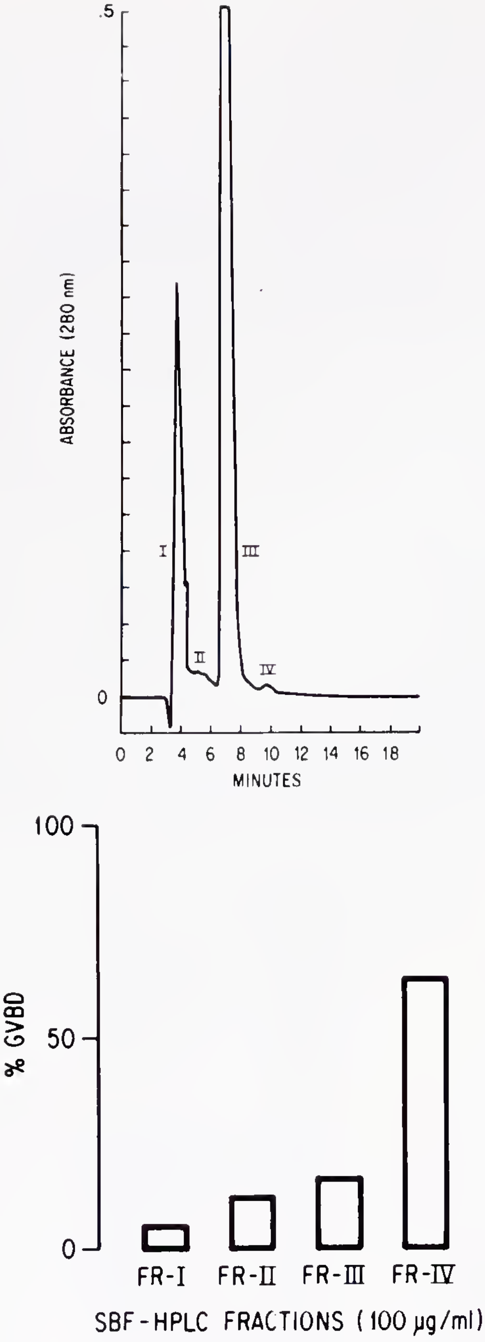

Figure 3. (A) HPLC elution profile of *Spisula* body fluid extract on reversed phase ODS-3 column (46 × 250 mm). The active factor was eluted with 0.1 *M* acetic acid at a flow rate of 1.0 ml/min. (B) GVBD-inducing activity of purified *Spisula* body fluid (SBF) separated by HPLC.

and reference 5-HT were analyzed separately by HPLC using a Bondapak C_{18} reverse-phase column (3.9 mm × 50). The HPLC was performed using an electrochemical detector system (Waters 460). A glassy carbon working electrode set at 0.650 V against the reference electrode was connected to the detector. The mobile phase consisted of 0.1 *M* citric acid, 75 m*M* Na_2HPO_4, 0.75 m*M* sodium 1-heptanesulfonate and 10% methanol (v/v) adjusted to pH 4.0 and delivered at a flow rate of 1 ml/min. The buffer was filtered (0.22 µm pore size) and degassed under vacuum just prior to use. All determinations were made at a sensitivity of 5 nA/V.

Spisula nerve ganglia: the nerve ganglia from each animal were excised and collected. Specimens from 10 animals were pooled. The pooled ganglia were suspended in ASW and homogenized by sonication. The homogenate was centrifuged at 3000 × *g* for 20 min at 4°C. The supernatant designated as *Spisula* nerve cell extract (SNE) was assayed for GVBD-inducing activity and purified as described above under Steps II and III.

Heat, trypsin, and charcoal treatment

Heat treatment was performed by placing the sample in boiling water at 100°C for 1 h. Digestion with trypsin was carried out by adding the protease (10 µg/ml) and incubating the mixture at 30°C for 1 h. Treatment with activated charcoal (30 mg/ml) was performed at room temperature for 1 h. Each procedure was performed separately and the treated samples assayed for maturation inducing activity.

Results

Spisula body fluid factor (SBF)

Crude SBF induced GVBD in 60% of the oocytes. The GVBD-inducing activity was potentiated in the presence of 5-HT. When SBF was subjected to ultrafiltration, the

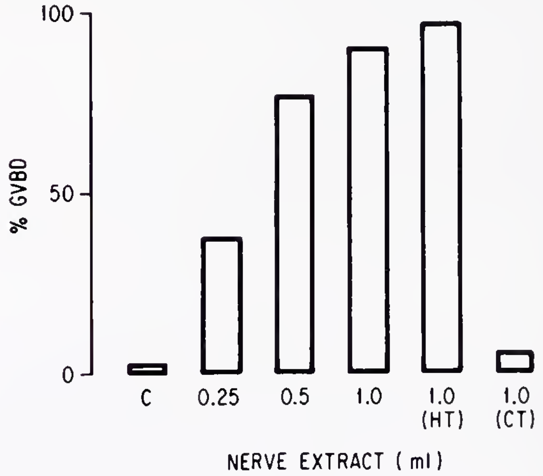

Figure 4. Dose-dependent induction of GVBD by *Spisula* nerve cell extract (SNE). After heat treatment (HT) and charcoal extraction (CT) SNE was assayed for GVBD-inducing activity as described under Materials and Methods.

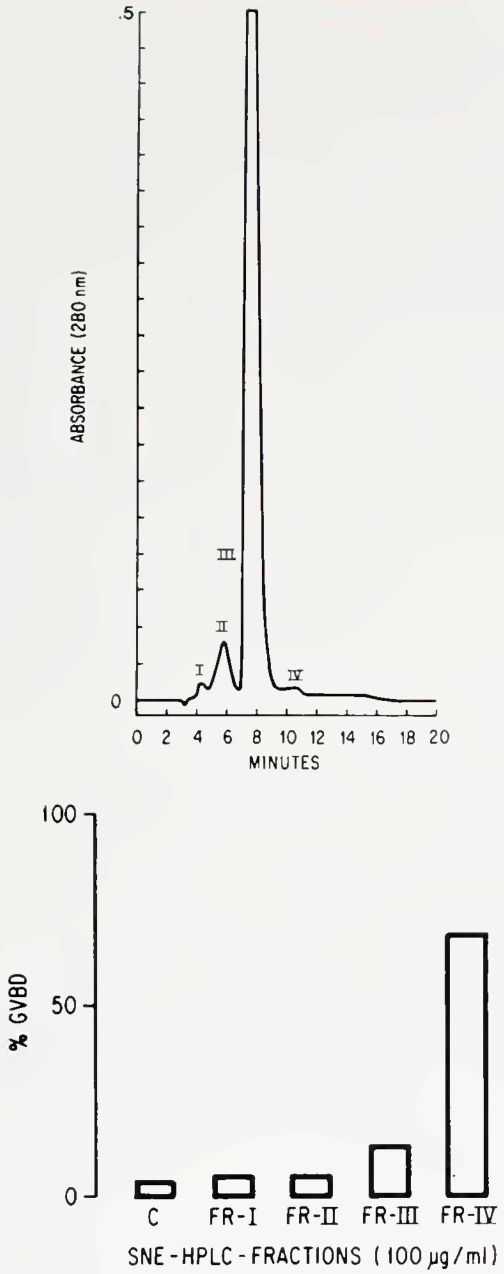

Figure 5. (A) Reversed phase HPLC elution profile of *Spisula* nerve cell extract (SNE). Eluting conditions were the same as described in Figure 3A. (B) GVBD inducing activity of HPLC-purified fractions of *Spisula* nerve cell extract (SNE). The four fractions separated by HPLC were assayed.

activity was located in the filtrate (PM-10-F). The filtrate alone or in the presence of 5-HT showed a dose-dependent GVBD-inducing activity (Fig. 1). When the filtrate (30 mg by weight per ml) was heated or treated with trypsin, the GVBD-inducing activity was retained. The activity in the filtrate was extracted by charcoal (Fig. 2) and lost on dialysis.

SBF extract was purified by HPLC. Four fractions were collected according to the elution profile (Fig. 3A). Fraction IV (FR-IV) possessed the greatest GVBD-inducing activity (Fig. 3B).

Spisula nerve ganglia factor (SNE)

SNE possessed potent, dose-dependent GVBD-inducing activity (Fig. 4). The activity was stable to heat and trypsin treatment. However, it was completely removed after charcoal treatment (Fig. 4). SNE was purified by HPLC and separated into four fractions (Fig. 5A). Only

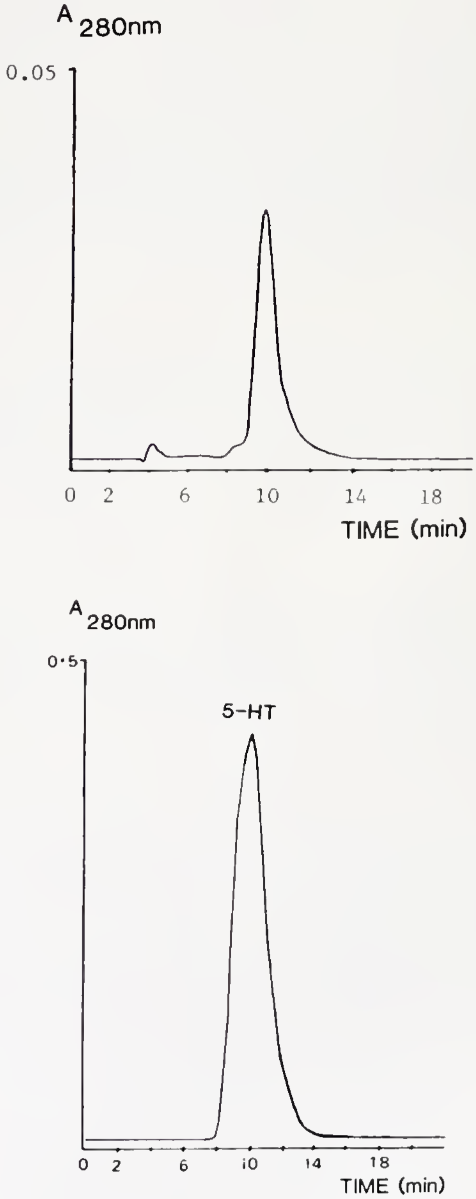

Figure 6. Reversed phase HPLC elution profile of the biologically active fraction (HPLC-FR-IV) of *Spisula* body fluid [A] and reference 5-HT [B]. Eluting condition was the same as described in Figure 5.

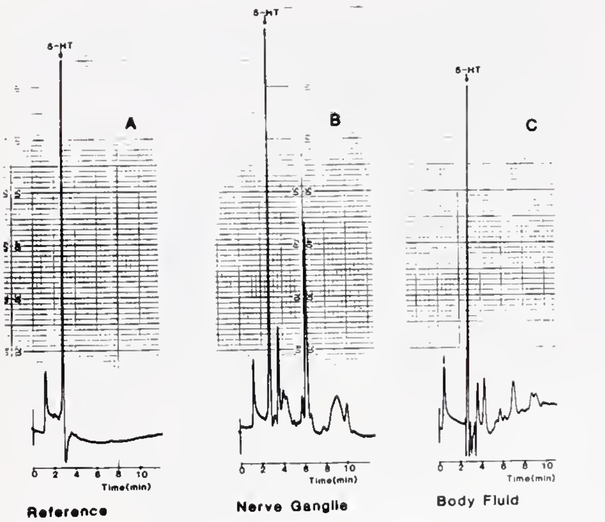

Figure 7. Isocratic separation of 5-HT utilizing HPLC with electrochemical detection. Representative elution profiles for (A) the reference 5-HT; (B) *Spisula* nerve ganglia extract (SNE); and (C) *Spisula* body fluid (SBF). The methods for HPLC and parameters for electrochemical detection are described in the text. Note detection of 5-HT peak in SNE and SBF.

fraction IV (FR-IV) showed OMI activity (Fig. 5B). It is noteworthy that the retention time of the substance isolated from nerve cells and body fluid and that of reference 5-HT analyzed by HPLC were the same, about 10 min (Fig. 6).

HPLC-ECD

With electrochemical detection, the HPLC profile of crude body fluid and nerve cell extracts show a peak coincident with 5-HT, *i.e.*, having the same retention time (2.5 min) as reference 5-HT (Fig. 7).

Discussion

In the present study a factor from *Spisula* body fluid and nerve cells was identified as 5-HT or a closely related compound. This conclusion is based on the findings that both the SBF substance and reference 5-HT which induced maturation had the same eluting retention time on analysis by HPLC and by electrochemical detection. The purified substance is probably 5-HT since the hormone has been found in the nervous system of bivalves (Welsh and Moorhead, 1960; Malanga *et al.*, 1972; Smith, 1982; Matsutani and Nomura, 1984). Both the SBF substance and 5-HT are stable to heat and trypsin treatment, and completely adsorbed by activated charcoal. They are not retained on dialysis in a tubing with a mol. wt. cut-off of less than 1000 daltons. Thus the SBF substance and 5-HT appear to be identical or closely related compounds. Also by HPLC-ECD, a peak coincident with reference 5-HT was found in the body fluid and ganglia extracts (Fig. 7). HPLC-ECD is used widely for the determination of 5-HT, its precursors, and metabolites in tissues (Trouvin *et al.*, 1987).

Maturation of *Spisula* oocytes can be induced by sperm on insemination, KCl, and other factors (Allen, 1953; Schuetz, 1975; Sato *et al.*, 1985; Toraya *et al.*, 1987). We emphasize that 5-HT injected into *Spisula* gonads induced spawning of sperm and oocytes (Hirai *et al.*, 1988). A majority of the spawned oocytes retained their germinal vesicles, suggesting that 5-HT may not induce maturation *in vivo*. However, we found recently that 8-hydroxy-2-(di-n-propylamino)-tetralin, a 5-HT_{1A} agonist (Middlemiss and Fozard, 1983), induced GVBD in *Spisula* oocytes (Kadam *et al.*, 1988). This suggests that *Spisula* oocytes possess 5-HT_{1A} receptor binding sites and the hormone may influence the metabolism of *Spisula* oocytes *in vivo*.

In the starfish, 1-methyladenine (1-MA) functionally resembles 5-HT (Kanatani, 1973). 1-MA triggers spawning and induces oocyte maturation in echinoderms. Both factors are neurotransmitters and have a profound effect on the gonads and oocytes. It is noteworthy that inducers of maturation are active on species-related oocytes. For example, progesterone induces maturation of amphibian oocytes (Masui and Clarke, 1979), 17α-hydroxy-20β-dihydroprogesterone with teleost oocytes (Suzuki *et al.*, 1981), and 1-MA with echinoderm oocytes (Kanatani, 1973). In *Chaetopterus*, an unidentified factor in natural seawater induces oocyte maturation (Ikegami *et al.*, 1976). Interestingly, oocyte maturation and spawning in *Spisula* and starfish are induced by small compounds. These substances are active only on species of the same family. The specific physiological inducers of spawning and oocyte maturation should be identified since this property may be a useful trait in the phylogenetic classification of species.

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Influence of Delayed Metamorphosis on the Growth and Metabolism of Young *Crepidula fornicata* (Gastropoda) Juveniles

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Abstract. Larvae of most marine invertebrates delay metamorphosis if they fail to contact an appropriate environmental stimulus. We conducted seven experiments with the slipper shell snail, *Crepidula fornicata,* to determine if delaying metamorphosis decreases juvenile fitness. Larvae were reared in the laboratory at 25°C on the unicellular alga *Isochrysis* sp. (clone T-ISO, 18×10^4 cells ml^{-1}), and were induced to metamorphose after long, medium, or short periods of delayed metamorphosis. Long delay larvae were reared until they metamorphosed spontaneously in acid-cleaned glassware. Medium and short delay larvae were induced to metamorphose with adult-conditioned seawater or 20 m*M* elevations of KCl. Juveniles were subsequently reared for about one to two weeks at 25°C in the laboratory on a diet of T-ISO. Delaying metamorphosis generally did not lower juvenile weight-specific feeding rates, increase juvenile weight-specific respiration rates, or lower juvenile shell or tissue growth rates, any of which effects would suggest that delaying metamorphosis reduces juvenile fitness. Moreover, there was no indication that delaying metamorphosis reduced juvenile tolerance of temperature-salinity stress. Delaying metamorphosis of *C. fornicata* does not appear detrimental, at least for the first few weeks of juvenile life.

Introduction

Many sedentary marine invertebrates have a free-swimming, dispersive larval stage (Thorson, 1950). Invertebrate larvae must generally develop for a time in the plankton before they become capable of metamorphosis. Once larvae are "competent," metamorphosis is commonly triggered by contact with chemical and physical cues characteristic of suitable adult habitat (Crisp, 1974; Gray, 1974; Scheltema, 1974; Hadfield, 1977; Jaccarini *et al.,* 1983; Olson, 1983; Sebens, 1983; Høeg, 1984).

If such cues are not encountered by competent larvae, metamorphosis may be delayed (*e.g.,* Knight-Jones, 1953; Wilson, 1958; Scheltema, 1961; Bayne, 1965; Hadfield, 1978; Pechenik, 1980). The ability to distinguish among substrates and to delay metamorphosis promotes preferential recruitment of larvae into habitats favorable for juvenile and adult survival and for reproduction (Thorson, 1950, 1966; Crisp, 1974). As a byproduct of this discriminatory capability, delaying metamorphosis enhances dispersal potential, which should influence a species' geographic range, the degree of genetic isolation among populations, rates of speciation, and species longevity (Shuto, 1974; Strathmann, 1974, 1978, 1980; Scheltema, 1977; Hansen, 1978; Beaumont and Budd, 1982; Jablonski and Lutz, 1983). This presumes that juvenile fitness (specifically, the probability of successfully giving rise to the next generation of reproductive adults) is not adversely affected by delayed metamorphosis (Pechenik, 1985). However, larvae of some invertebrate species exhibit morphological degeneration while they delay metamorphosis (Wilson, 1935; Bayne, 1965; Sastry, 1965; Hinegardner, 1969; Highsmith and Emlet, 1986) and may actually lose the ability to metamorphose in response to suitable substrates after a lengthy delay period (Wilson, 1935; Grave, 1936; Knight-Jones, 1953; Hinegardner, 1969; Barker, 1977; Sebens, 1983). Other delaying larvae, including those of the gastropod *Crepidula fornicata,* that fail to show such dramatic morphological and physiological degeneration during delayed metamorphosis (Pechenik, 1980, 1984) may nevertheless experience subtle declines in fitness that could be car-

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ried into juvenile life. Any such decrease in fitness would lessen the importance of delay capability in maintaining genetic homogeneity and influencing rates of extinction and speciation.

Characterizing such evolutionary "design constraints" is a major goal of life history research (Stearns, 1980; Reznick, 1982). The possibility that fitness is reduced following an extended delay of metamorphosis is readily testable for *C. fornicata* in the laboratory. Reduced fitness could result in higher juvenile mortality, decreased rates of energy acquisition, increased rates of energy utilization, delayed maturation, reduced fecundity, or reduced tolerance to physical and biological stress (Stearns, 1976; Bayne, 1975; Newell and Branch, 1980; Berven, 1982). This report focuses on the influence of delayed metamorphosis on survival, energy accumulation, and tolerance of physical stress by juveniles of *Crepidula fornicata*.

Crepidula fornicata is a rapidly growing (Calabrese and Rhodes, 1974), sedentary, suspension-feeding gastropod. These characteristics facilitate measuring growth, respiration, and feeding rates. Moreover, the larvae of this species can be laboratory-reared with very low mortality (Pechenik, 1980; Pechenik and Lima, 1984), competent larvae can be readily induced to metamorphose (Pechenik and Heyman, 1987), and even young juveniles are large enough to allow accurate determinations of individual dry tissue weights. Finally, *C. fornicata* is one of the few temperate molluscs whose larvae have been demonstrated to delay metamorphosis in the field (Pechenik, 1986).

Materials and Methods

Obtaining and rearing animals

Adult *Crepidula fornicata* were collected from Narragansett, Rhode Island (1985), Barnstable, Massachusetts (1986), and Nahant, Massachusetts (1987) and fed the unicellular flagellate *Dunaliella tertiolecta* daily until veliger larvae were released from the brooded egg cases. We reared larvae at 25°C in Percival incubators (12L:12D photoperiod), with about one larva per ml of 0.45 μm filtered seawater, and fed them the alga *Isochrysis* sp. (clone T-ISO; Bigelow Laboratory Culture Collection) at $18–20 \times 10^4$ cells ml^{-1}. Water was changed and food was replenished every other day. Seawater was aerated by vigorous agitation just before adding larvae and phytoplankton, but not between water changes.

About 10 days after hatching, larvae were measured at 50× using a dissecting microscope fitted with an ocular micrometer. Larvae with shell lengths between 800 and 1200 μm were segregated by pipette and randomly divided into two groups; Pechenik and Heyman (1987) demonstrated that most *C. fornicata* larvae can metamorphose by the time they are 800–1000 μm long. Larvae in one group were triggered to metamorphose over the next 6–8 h using either adult-conditioned seawater or seawater whose KCl concentration was elevated by 20 m*M* (Pechenik and Heyman, 1987); Eyster and Pechenik (1988) showed that shell growth, weight-specific respiration, and weight-specific feeding rates of young juveniles are comparable following both modes of induction. These larvae are referred to as "short delay" individuals, since they probably had delayed metamorphosis for less than two days.

Larvae in the second group were maintained until each individual metamorphosed "spontaneously." By spontaneous metamorphosis, we refer to metamorphosis that eventually occurs despite our best efforts to prevent it; larvae of *C. fornicata* inevitably undergo such spontaneous metamorphosis in laboratory culture despite daily cleaning of glassware (Pechenik, 1984; Pechenik and Lima, 1984). These juveniles were designated "long delay" individuals, resulting from the maximum possible extent of delayed metamorphosis.

In two experiments, we reared three groups of juveniles: short delay individuals (as above), long delay individuals (as above), and medium delay individuals (triggered to metamorphose using KCl 4–6 days after metamorphosis was triggered in short delay individuals).

All juveniles were reared at 25°C for 6–13 days in individual glass dishes containing 45 ml of T-ISO feeding suspension at an initial concentration of 18×10^4 cells ml^{-1}. Initial shell lengths were measured at 50×, usually within 12 h of metamorphosis. Feeding suspension in each bowl was changed daily, but algal cell density nevertheless declined markedly between water changes. Therefore, we conducted an experiment to estimate the phytoplankton cell density below which feeding rates decline for juveniles of *C. fornicata*. Preliminary studies (Eyster and Pechenik, 1988) suggested that this critical cell density was about 8×10^4 cells ml^{-1} for 1.7 mm long juveniles feeding on T-ISO.

Determining critical food concentration

To determine the critical phytoplankton cell concentration for recently metamorphosed juveniles, 24 juveniles (mean shell length ± one standard deviation = 2.84 ± 0.33 mm) were selected for the experiment. Anterior-posterior shell lengths were measured at 25× using an ocular micrometer. All juveniles were allowed to pre-feed for 30 min at 18×10^4 T-ISO cells ml^{-1} in individual 5-ml glass beakers. The pre-feeding suspension was then replaced with 4 ml of suspension at the same algal concentration, 18×10^4 cells ml^{-1}. After 1 h at 25°C, the final cell concentrations were determined in all 24 beakers. Cell concentrations were also determined in five controls at the start of the experiment and five controls at the end of the experiment. All incubations were conducted in dim light. Cell concentrations in control beakers did not change appreciably during this experiment. All cell concentrations were determined using a Model ZM electronic particle counter (Coulter Electronics), and occasionally double-checked using hemacytometers. Feeding rates were determined from the rate of disappearance of cells from suspension (Pechenik, 1980; Marin *et al.*, 1986; Eyster and Pechenik, 1988).

After feeding rates were determined for all individuals fed at 18×10^4 cells ml^{-1}, feeding suspensions were replaced in all beakers. Twelve animals were repeatedly fed at 18×10^4 cells ml^{-1}. The other 12 animals were fed at progressively lower initial food levels for each one-hour feeding bout. Animals in the two groups did not differ in mean shell length ($P < 0.05$; one-way analysis of variance). The following algal concentrations were tested: 18×10^4, 11×10^4, 9×10^4, 7×10^4, and 4×10^4 cells ml^{-1}. Feeding rates were then again determined for all 24 individuals at an initial concentration of 18×10^4 cells ml^{-1}.

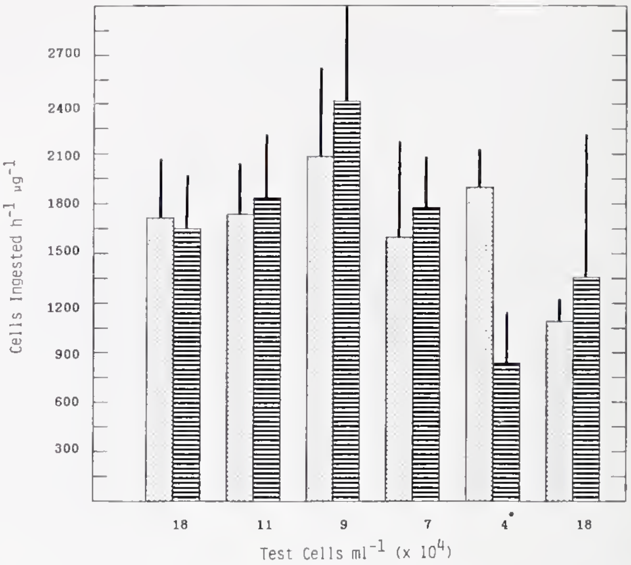

Figure 1. The influence of algal cell concentration (*Isochrysis* sp., Tahitian isolate) on weight-specific ingestion rate of juvenile *Crepidula fornicata* One group of 12 juveniles (dotted bars) was fed at an initial cell concentration of 18×10^4 cells ml^{-1} in each 1-h feeding bout. The other group of 12 juveniles (striped bars) was fed at progressively lower food concentrations every 1 h as indicated. To complete the experiment (extreme right), both groups of snails were offered 18×10^4 cells ml^{-1}. *Represents a significant difference between means ($P < 0.05$, one-way analysis of variance). Error bars indicate one standard deviation above the mean.

Effects of delayed metamorphosis on growth and metabolism

We performed six experiments testing the influence of delayed metamorphosis on juvenile development. Larvae were induced to metamorphose after different amounts of delayed metamorphosis, as detailed above. Rates of individual juvenile shell growth were then monitored by periodic microscopic examination. Three to 13 days after triggering metamorphosis, we carefully transferred the juveniles to individual 5 ml glass bottles to measure individual respiration and feeding rates at 25°C, as described elsewhere (Eyster and Pechenik, 1988). After a 1–2 h gut evacuation period in 0.45 µm filtered seawater, respiration rates were determined from the decline in oxygen concentration over 4–15 h periods, the length of each incubation period depending on juvenile size. Oxygen concentrations were determined by injecting 1-ml samples into a Strathkelvin Instruments Model MC100 glass microrespiration cell equipped with a Model 1302 electrode; the electrode was coupled to a Strathkelvin Model 781 oxygen meter. Individual feeding rates were determined from declines in algal cell concentration over 1–5 h periods, the experimental duration again varying inversely with juvenile size.

After measuring respiration and feeding rates, we measured the final shell length of each snail and then determined the individual's ash free dry weight (AFDW). This enabled us to calculate weight-specific respiration and feeding rates. To obtain dry weights, each juvenile was removed from its test container, measured at 25×, rinsed free of salts, transferred to preweighed aluminum foil pans, and desiccated over indicating $CaSO_4$ for approximately one week. Individual dry weights were determined to the nearest 1 µg using a Cahn Model 21 electrobalance, with desiccant placed in the weighing chamber to prevent specimen rehydration. Samples were then ashed at 500°C for 6 h and reweighed to estimate tissue content by weight loss (Paine, 1964; Pechenik, 1980).

Linear regression analysis (method of least squares) was used to determine the relationship between final juvenile shell length (mm) and tissue weight (µg); this enabled us to estimate weight at metamorphosis from shell length data, and to express metabolic data on a weight-specific basis when direct tissue weight measurements were not available, due to loss or mishandling. In one experiment (No. 10), juvenile shell length measurements were made twice: once on day 7 and later on day 13, at which time actual individual weight determinations were made. Thus, we could calculate shell growth rate for the first 7 days of juvenile development, the first 13 days of juvenile development, and between days 7–13. The regression equation was used to estimate individual biomass on day 7 so that tissue growth rates could be calculated separately for the first and second weeks of juvenile growth.

Effects of delayed metamorphosis on stress tolerance

In addition to examining the influence of delayed metamorphosis on juvenile growth and metabolism, we

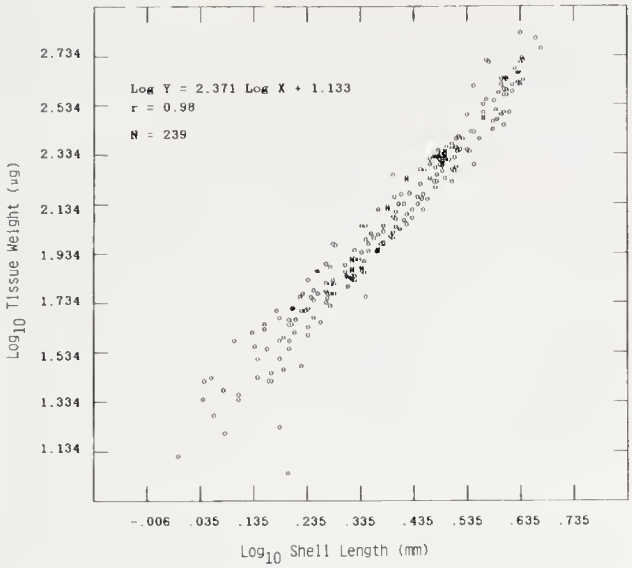

Figure 2. The relationship between tissue weight and longest shell length for juvenile *Crepidula fornicata*. Tissue weights were estimated as ash-free dry weight after combustion at 500°C. Each point represents data from a single individual.

also conducted experiments to determine whether delayed individuals were less tolerant of physical stress than were individuals triggered to metamorphose soon after they became competent. Larvae reared at 25°C were divided into two groups: short delay individuals were triggered to metamorphose soon after they developed shell brims (Pechenik, 1984; Pechenik and Lima, 1984); long delay individuals were either triggered to metamorphose about one week later or were reared through spontaneous metamorphosis. All juveniles were reared at 25°C for 5–7 days at about 18 × 10^4 cells ml^{-1} on a mixed diet of T-ISO and *Dunaliella tertiolecta* (clone DUN). They were then distributed among combinations of five osmotic concentrations (460, 610, 770, 830, and 925 mOsm kg^{-1}—approximately 15, 20, 25, 27, and 30 ppt salinity, with full strength seawater being the highest concentration) and five temperatures covering the range 25°–35°C. Osmotic concentrations were determined using a freezing-point depression osmometer (Advanced Instruments, Inc). Test solutions were prepared by diluting full strength Nahant-collected seawater with glass-distilled water. Five to eight juveniles were tested at each stress level, without replication, and examined after 24 and 36 h of exposure. Tested juveniles were 1–2 mm in shell length. Four separate stress experiments were conducted, each using juvenile snails from separate hatches.

Results

Critical food concentration

Juvenile weight-specific feeding rates were unaffected by phytoplankton cell concentrations between about 7 and 18 × 10^4 cells ml^{-1}. However, decreasing cell concentration to 4 × 10^4 cells ml^{-1} caused a 56% reduction in average feeding rate (Fig. 1). Clearly, the critical food level for these juveniles lies somewhere between 4 and 7 × 10^4 cells ml^{-1}, confirming previous indications (Eyster and Pechenik, 1988). Spot checks indicated that algal cell concentrations rarely declined below about 8–10 × 10^4 cells ml^{-1} during our juvenile growth studies. This suggests that juveniles were not food limited during our study and that growth rates among these juveniles can be reasonably compared.

Survival

Juveniles survived well following metamorphosis. Of the 320 juveniles studied in these experiments, only about 12% died; one-third of these died only because they crawled out of the water and desiccated. The incidence of this "suicidal" behavior was unrelated to the extent of delayed metamorphosis.

Table I

Influence of delayed larval metamorphosis on subsequent juvenile weight-specific respiration rates in Crepidula fornicata

	Mean respiration rates (µl O_2 consumed h^{-1} µg $tissue^{-1}$) × 10^{-3}				
Treatment	Expt. 3	Expt. 4	Expt. 5	Expt. 6	Expt. 9
Short delay	3.6 ± 0.6 (17)	2.7 ± 0.5 (27)	2.8 ± 0.5 (20)	3.6 ± 0.5 (18)	3.5 ± 0.85 (26)
Medium delay	NT	NT	NT	4.1 ± 0.9 (13)	NT
Long delay	2.8 ± 0.5 (7)	3.6 ± 0.8 (7)	2.1 ± 0.7 (8)	3.5 ± 0.5 (11)	3.9 ± 1.2 (10)
ANOVA F-value	F = 8.72*	F = 13.30*	F = 9.23*	F = 2.87	F = 1.61
(degrees of freedom)	(1, 22)	(1, 32)	(1, 26)	(2, 39)	(1, 34)

Short delay and medium delay animals were induced to metamorphose using adult-conditioned seawater or elevated levels of KCl. Long delay individuals metamorphosed spontaneously. Rates are mean ± one standard deviation (sample size). * = Significant difference exists among treatments, ANOVA, $P < 0.05$. NT = Not tested.

Table II

Influence of delayed metamorphosis on subsequent weight-specific feeding rates of Crepidula fornicata *juveniles*

Treatment	Mean feeding rates (cells ingested h^{-1} μg tissue^{-1}) × 10^3					
	Expt. 3	Expt. 4	Expt. 5	Expt. 6	Expt. 9	Expt. 10
Short delay	1.46 ± 0.55 (17)	1.06 ± 0.18 (27)	1.54 ± 0.68 (20)	0.86** ± 0.17 (19)	0.73 ± 0.35 (25)	0.86 ± 0.26 (13)
Medium delay	NT	NT	NT	1.26 ± 0.23 (14)	NT	1.36** ± 0.23 (18)
Long delay	1.36 ± 0.58 (8)	0.93 ± 0.48 (7)	1.75 ± 0.67 (8)	1.11 ± 0.24 (11)	0.75 ± 0.26 (10)	0.94 ± 0.23 (16)
ANOVA F-value (degrees of freedom)	F = 0.16 (1, 23)	F = 1.22 (1, 32)	F = 0.56 (1, 26)	F = 14.80* (2, 41)	F = 0.04 (1, 33)	F = 20.19* (2, 44)

Rates are mean ± one standard deviation (sample size). See Table I for treatment details. * = Significantly different by ANOVA, $P < 0.05$. ** = Treatment that is significantly different by Duncan's multiple range test, $P < 0.05$. NT = Not tested.

Weight-length relationship

The relationship between juvenile tissue weight and shell length in *C. fornicata* was well defined by the following equation: $\log_{10}$ μg tissue = 2.371 ($\log_{10}$ mm shell length) + 1.133 (N = 239; $r^2 = 0.96$) (Fig. 2). Therefore, the units of growth rate could be converted from amount of shell added per day to μg tissue added per day using this equation.

Effect of delayed metamorphosis on respiration and feeding

Weight-specific respiration rates were significantly different ($P < 0.05$) for long and short delay individuals in three of the five experiments conducted. They were significantly higher in only one experiment (experiment 4) (Table I). Weight-specific feeding rates were not significantly lower ($P > 0.10$) for long delay individuals in any of the six experiments (Table II); significant differences in mean feeding rates were recorded in two experiments, but these differences reflect either higher mean feeding rate in medium and long delay snails (experiment 6) or higher mean feeding rate in medium delay snails (experiment 10).

Effect of delayed metamorphosis on juvenile growth

Determining the influence of delayed metamorphosis on juvenile growth rate is potentially complicated by an unavoidable difference in the sizes at which individuals metamorphose in the different treatments. Long delay individuals were typically larger at metamorphosis (Table III), since *C. fornicata* larvae continue to grow as metamorphosis is delayed (Pechenik, 1980, 1984; Pechenik and Lima, 1984). This size difference was not a problem when comparing postmetamorphic shell growth, since rates of juvenile shell growth were not correlated ($P > 0.10$) with shell size at metamorphosis. Since no more than 10% of the variation in individual juvenile shell growth could be accounted for by differences in shell length at metamorphosis ($r^2 < 0.10$, Table IV), shell growth rates for snails in the different treatment groups

Table III

Shell sizes (mean μm ± one standard deviation) at metamorphosis for Crepidula fornicata *larvae delaying metamorphosis for different lengths of time*

Treatment	Expt. 3	Expt. 4	Expt. 5	Expt. 6	Expt. 7	Expt. 10
Short delay	1343 ± 187 (N = 17)	1118 ± 207 (N = 28)	1234 ± 141 (N = 20)	1100 ± 149 (N = 20)	891 ± 128 (N = 20)	942 ± 165 (N = 13)
Medium delay	NT	NT	NT	1075 ± 108 (N = 14)	NT	982 ± 123 (N = 18)
Long delay	1541 ± 186 (N = 8)	1311 ± 163 (N = 7)	1550 ± 166 (N = 8)	1189 ± 204 (N = 11)	1256 ± 109 (N = 12)	1202** ± 217 (N = 16)
ANOVA F-value (degrees of freedom)	F = 6.1* (1, 23)	F = 5.2* (1, 32)	F = 25.9* (1, 26)	F = 1.8 (2, 42)	F = 67.4* (1, 30)	F = 10.3* (2, 44)

Metamorphosis was triggered using adult-conditioned seawater or 20 m*M* elevations of KCl for "short delay" and "medium delay" animals; "long delay" individuals were allowed to metamorphose spontaneously. * and ** = Significant differences ($P < 0.05$), ANOVA and Duncan's multiple range test, respectively. NT = Not tested.

Table IV

Extent of correlation between Crepidula fornicata *juvenile growth rate and initial postmetamorphic shell length*

Experiment	N	Shell growth rate (μm d^{-1}) vs initial shell length (μm)	Tissue growth rate (μg d^{-1}) vs initial shell length (μm)
3	45	−0.04 (0.002)	0.39* (0.15)
4	34	−0.23 (0.05)	0.44* (0.20)
5	29	0.16 (0.03)	0.53* (0.28)
6	45	−0.04 (0.002)	0.39* (0.15)
7	32	0.31 (0.10)	0.69* (0.48)
10a	47	0.18 (0.03)	0.53* (0.28)
10b	47	−0.03 (0.001)	0.53* (0.28)

Data are presented as r(r^2). * = Significant correlation ($P < 0.05$).

could be compared directly, despite the size differences at metamorphosis. Rates of shell growth were significantly reduced ($P < 0.05$) for delayed individuals in only two of the seven determinations (experiments 4 and 10b); they were actually higher ($P < 0.05$) in one case (experiment 7) (Table V).

In contrast to results for shell growth, tissue growth rates correlated significantly ($P < 0.05$) with shell size at metamorphosis in all experiments (Table IV). That is, individuals that were larger at metamorphosis tended to add biomass more rapidly following metamorphosis (Fig. 3). Thus, direct comparison of tissue growth rate among treatment groups could be misleading: any tendency of long delay individuals to add biomass more slowly than short delay individuals could be masked by the larger average size of the long delay animals at metamorphosis. Therefore, mean tissue growth rates within an experiment were adjusted by analysis of covariance, with shell length at metamorphosis serving as the covariate.

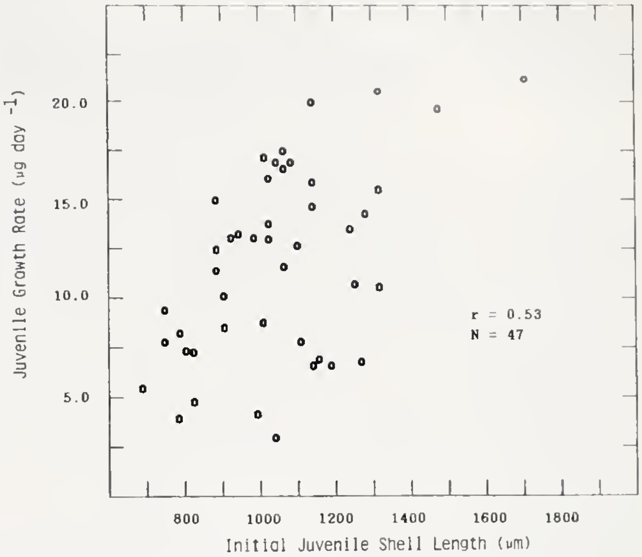

Figure 3. Representative relationship between size at metamorphosis and daily tissue growth in juvenile *Crepidula fornicata.* These data are from experiment 10a, in which juveniles grew for 7 days following metamorphosis.

Without adjustment, long delay individuals added biomass more slowly than medium or short delay individuals in only one of the six experiments (experiment 4) (Table VI). Adjusting for differences in initial shell lengths among treatment groups, long delay individuals grew significantly more slowly than other individuals in two experiments (experiments 4 and 10) (Table VI).

The data for experiment 10 are susceptible to separate analysis since juvenile size was measured thrice, on days 1, 7, and 13 after metamorphosis. Thus, juvenile growth rates can be examined separately for the first and second weeks of development. Although at metamorphosis shell sizes (and biomass) differed among treatment groups in experiment 10 (Table III), these differences had eva-

Table V

Influence of delayed larval metamorphosis on shell growth rate of juvenile Crepidula fornicata

Treatment	Expt. 3	Expt. 4	Expt. 5	Expt. 6	Expt. 7	Expt. 10a	Expt. 10b
	Growth rates: mean μm day^{-1} ± one standard deviation (sample size)						
Short delay	126 ± 31 (18)	208 ± 24 (28)	183 ± 43 (21)	201 ± 39 (20)	220 ± 43 (20)	167 ± 30 (13)	200 ± 31 (13)
Medium delay	NT	NT	NT	195 ± 37 (14)	NT	188 ± 24** (18)	194 ± 32 (18)
Long delay	150 ± 46 (8)	141 ± 51 (6)	173 ± 13 (8)	166 ± 37 (11)	256 ± 28 (12)	150 ± 53 (16)	146 ± 30** (16)
ANOVA F-value (degrees of freedom)	F = 2.31 (1, 23)	F = 25.2* (1, 32)	F = 1.64 (1, 27)	F = 2.39 (2, 41)	F = 6.56* (2, 29)	F = 7.18* (2, 44)	F = 9.86* (2, 44)
Number of days	5	3–4	5–6	4–5	9	7	13

Larval treatments are given in Table I. Juvenile growth rates were compared over 3–13 days as indicated. * = Significantly different by ANOVA, $P < 0.05$. ** = Significantly different by Duncan's multiple range test, $P < 0.05$. NT = Not tested.

Table VI

Influence of delayed metamorphosis on tissue growth in juvenile Crepidula fornicata

	Growth rate: mean μg tissue day^{-1} ± one standard deviation (sample size)						
Treatment	Expt. 3	Expt. 4	Expt. 5	Expt. 6	Expt. 7	Expt. 10a	Expt. 10b
Short delay	8.0 ± 2.4 (18)	11.8 ± 3.2 (28)	13.1 ± 4.6 (21)	11.6 ± 3.8 (20)	24.5 ± 8.4 (20)	10.1 ± 3.9 (13)	20.3 ± 7.4 (13)
Medium delay	NT	NT	NT	11.1 ± 3.1 (14)	NT	13.3 ± 3.5 (18)	20.7 ± 6.3 (18)
Long delay	11.7 ± 4.7 (7)	8.3 ± 3.2 (6)	14.6 ± 2.4 (8)	9.7 ± 3.2 (11)	39.1 ± 7.0 (12)	11.0 ± 6.1 (16)	17.2 ± 9.4 (16)
ANOVA F-value	6.93*	6.02*	0.74	1.08	25.7*	1.92	0.96
(degrees of freedom)	(1, 23)	(1, 32)	(1, 27)	(2, 42)	(1, 30)	(2, 44)	(2, 44)
Adjusted means							
short delay	8.0	12.2	14.6	11.8	27.0	12.1	23.4
medium delay	NT	NT	NT	11.5	NT	14.5	22.6
long delay	11.1	6.3	10.7	8.9	35.0	8.0	12.5
F-value:	3.4	27.9**	3.2	3.1	4.28**	12.4**	11.5**
Adjusted by ANCOVA							
Number of days	5	3–4	5–6	4–5	9	7	13

Larval treatments are given in Table I. Tissue weights at metamorphosis were estimated from shell length data using the relationship illustrated in Fig. 2. * = Significant differences ($P < 0.05$) by one-way analysis of variance (ANOVA) or by analysis of covariance (**) (ANCOVA). ANCOVA was performed with initial postmetamorphic shell length as the covariate, to adjust for differences in mean size at metamorphosis among treatment groups within an experiment. NT = Not tested.

nesced by day 7, as did any differences in mean tissue weight (Table VII). Therefore, mean rate of tissue growth can be compared for the subsequent 6 days of juvenile growth without data adjustment. Delaying metamorphosis had no effect on rate of shell or tissue growth for the second week following metamorphosis (Table VII).

In all experiments juveniles grew rapidly following metamorphosis. Shell growth rates averaged about 150–200 μm day^{-1}, the equivalent of accumulating biomass at about 10–20 μg dry weight day^{-1} (Tables V, VI).

Effect of delayed metamorphosis on stress tolerance

In the temperature-salinity stress study, mortality was always 100% at 35°C (all salinities). Mortality was always 0% at 25°C and 30°C (all salinities). None of our experiments indicated that delaying metamorphosis reduced tolerance of *C. fornicata* to temperature-salinity stress. Indeed, the data for 24 h (Table VIII) and 36 h (Table IX) survival suggest that long delay individuals are more tolerant of stress than short delay individuals.

Discussion

Delaying the metamorphosis of the gastropod *Crepidula fornicata* typically had no detectable detrimental influence on juvenile survival, feeding rate, respiration rate, shell growth rate, tissue growth rate, or tolerance to temperature-salinity stress for at least the first few weeks following metamorphosis (Tables IX, X). Similarly, de-

Table VII

The lack of influence of delayed metamorphosis on juvenile growth rates of Crepidula fornicata *from days 7–13 after metamorphosis in experiment 10*

	N	Actual 7d shell length (mm)	Estimated 7d tissue wt. (μg)	Actual 13d tissue wt. (μg)	Tissue growth rate (μg d^{-1})
Short delay	13	2.11 ± 0.35	83.3 ± 31.4	275.7 ± 100.1	32.0 ± 12.6
Medium delay	18	2.36 ± 0.26	106.0 ± 26.7	282.2 ± 83.3	29.4 ± 10.3
Long delay	16	2.24 ± 0.50	99.1 ± 50.6	245.9 ± 130.0	24.5 ± 16.1
F-value (2, 44)		1.60	1.40	0.54	1.32
P-value		0.21	0.26	0.58	0.28

Data are given as mean ± one standard deviation.

Table VIII

Percent mortality of juvenile Crepidula fornicata *exposed to various salinity-temperature combinations for 24 h*

			Percent morality Salinities				
Experiment	Length of delay	Temp °C	15‰	20‰	25‰	27‰	30‰
A, B, C, D	short, long	25°	0	0	0	0	0
A, B, C, D	short, long	30°	0	0	0	0	0
A, B, C, D	short, long	35°	100	100	100	100	100
A	short	32°	100	0	0	—	—
	long		100	0	0	—	0
A	short	33.5°	100	100	60	—	—
	long		100	100	60	—	60
B	short	32°	**80**	0	0	—	—
	long		**0**	0	0	—	0
B	short	33.5°	100	100	100	—	—
	long		100	100	100	—	100
C	short	32°	0*	0	0	—	0
	long		0*	0	0	—	0
C	short	33.5°	100	100	100	—	0*
	long		100	100	100	—	0*
D	short	32°–32.8°	100	0*	**0***	—	**25**
	long	32°–33.2°	100	0*	**0**	—	**0**
D	short	33.5°	100	100	100	100	100
	long		100	100	100	100	100

— = Not tested. * = None dead but all moribund. Experiments where mortality varied between short delay and long delay treatments are shown in boldface.

laying metamorphosis did not significantly alter juvenile growth rates for the sand dollar *Dendraster excentricus* (Highsmith and Emlet, 1986, p. 350 and their fig. 4). Highsmith and Emlet (1986) also worked with the sand dollar *Echinarachnius parma* and found that delaying metamorphosis rarely depressed juvenile growth rates

Table IX

Percent mortality of juvenile Crepidula fornicata *exposed to various salinity-temperature combinations for 36 h*

			Percent mortality Salinities				
Experiment	Length of delay	Temp °C	15‰	20‰	25‰	27‰	30‰
A	short	32°	100	**100**	**50**	—	—
	long		100	**0**	**0**	—	0
A	short	33.5°	100	100	100	—	—
	long		100	100	100	—	60
B	short	32°	**100**	0	0	—	—
	long		**25**	0	0	—	0
C	short	32°	**100**	0	0	—	0
	long		**0***	0	0	—	0
C	short	33.5°	100	100	100	—	100
	long		100	100	100	—	100
D	short	32°–32.8°	100	**25**	**40**	—	**25**
	long	32°–33.2°	100	**40**	**0**	—	**0**

— = Not tested. * = None dead but all moribund. As in 24 h exposures, mortality was 0% at 25°C and 30°C (all salinities, even for 48 h). Those experiments in which mortality was already 100% in all treatments by 24 h are not relisted in this table (see Table VIII). Experiments where mortality varied between short delay and long delay treatments are shown in boldface.

Table X

Summary table: effects of delayed metamorphosis on juvenile growth and metabolism in Crepidula fornicata

	Number of experiments showing increase in delayed individuals	Number of experiments showing decrease in delayed individuals	Number of experiments showing no effect
Shell growth rate	1	2	4
Tissue growth rate	2	1	4
Adjusted tissue growth rate	1	3	3
Weight-specific feeding rate	1	0	5
Weight-specific respiration rate	1	2	2

In this table, determinations 10a and 10b are considered separate experiments. Summary is based on comparisons of short delay versus long delay individuals only.

(their Table 6, p. 356). Moreover, they found no correlation between juvenile growth rate and the number of days that metamorphosis was delayed. Their data for *D. excentricus* suggest a possible relationship between extent of delayed metamorphosis and rate (but not necessarily extent) of juvenile mortality. However, their results may reflect inadequate culture conditions more than a direct effect of delayed metamorphosis on survival: the water in larval cultures was changed only once or twice per week, the water in juvenile cultures was changed as seldom as once every two weeks, larvae and juveniles were fed unknown amounts of food as infrequently as once per week, and unfed juveniles showed dramatically less mortality than fed individuals from the same experiments. If the substantial mortality Highsmith and Emlet (1986) report for juvenile *D. excentricus* does reflect inhospitable culture conditions, then, as the authors suggest, delaying metamorphosis appears to have made those juveniles more susceptible to that stress. Of those individuals that died, long delay animals tended to die before short delay animals. In contrast, our experiments suggest that juvenile *C. fornicata* may actually be more tolerant of physical stress if metamorphosis has been delayed.

In contrast to these results for *C. fornicata*, we recently found that delaying metamorphosis of the bryozoan *Bugula stolonifera* by only 8–10 h significantly reduced the rate of early colony development (Woollacott *et al.*, in press). Unlike the gastropod and echinoid larvae discussed above, the bryozoan larvae are non-feeding, and the impact of delaying metamorphosis likely reflects depletion of stored nutrients. Since *C. fornicata* larvae continue to feed while metamorphosis is delayed (Pechenik, 1980; Pechenik and Lima, 1984), delaying metamorphosis is unlikely to impose nutritional stress in this species.

Critical food concentrations for the early juveniles (about 2–3 mm shell length) feeding on the alga *Isochrysis* sp. (clone T-ISO) were between 4–7 × 10^4 cells ml^{-1}, confirming preliminary studies with slightly smaller juveniles of this species (Eyster and Pechenik, 1988). Because we usually succeeded in maintaining food levels above about 8 × 10^4 cells ml^{-1} between water changes in our experiments, we can assume that all juveniles were growing at their maximal rates and that juvenile growth rates were independent of food level. Pechenik (1978) reported a higher critical food concentration—somewhere between 10–12 × 10^4 cells ml^{-1}—for larvae of this species.

The growth rates we report here (150–200 μm day^{-1}) for juveniles of *C. fornicata* are somewhat higher than those reported earlier for juveniles reared in this laboratory at the same temperature and on the same diet (Pechenik and Lima, 1984). The maximum growth recorded previously at 25°C was about 130 μm day^{-1}. Our data demonstrating rapid growth in this species are consistent with earlier reports of rapid development; sexual maturity in *C. fornicata* is reached, in the laboratory at 25°C, in about only 105 days after metamorphosis (Calabrese and Rhodes, 1974).

Comparable metabolic data for juvenile *C. fornicata* are available only for much larger individuals (20–40 mm long; Newell and Kofoed, 1977). At 25°C, weight-specific respiration rates of these larger snails were typically one-half to one-quarter the values reported here for individuals only a few mm long.

Pechenik (1985) suggested that a gradual decline in fitness could explain why natural selection might not act to prolong larval life indefinitely in *C. fornicata*. It is therefore puzzling that spontaneous metamorphosis occurs in the absence of a demonstrable cost to delaying metamorphosis. Although we found no consistent negative influence of delayed metamorphosis on early juvenile development, we cannot rule out the possibility that negative effects on survival, growth, or metabolism appear in later development. Other potential costs of delaying metamorphosis include effects on fecundity, age at first reproduction, and resistance to predation. These have not yet been investigated for any marine invertebrate.

The results of this study have implications for molluscan mariculture. Molluscan larvae develop at widely different rates even within a single culture (*e.g.*, Hadfield, 1977; Newkirk *et al.*, 1977; review by Pechenik, 1987). Therefore, when most of the larvae in a culture are competent, many of the larvae in that culture will have already been competent to metamorphose for some time.

Based on this work for *C. fornicata*, it appears that stimulating metamorphosis after a lengthy delay period will not reduce juvenile survival or growth, at least for species with feeding larvae. Even so, similar experiments on commercially important molluscan species should be conducted before generalizations are made.

Acknowledgments

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Existence and Functions of a Gel Filled Primary Body Cavity in Development of Echinoderms and Hemichordates

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Abstract. An extensive gelatinous material occupies the primary body cavity of larval echinoderms (auricularia, bipinnaria, ophiopluteus, and echinopluteus) and hemichordates (tornaria). Its presence and its recovery of shape following application and release of force were demonstrated by dissection of larvae in a suspension of sumi ink. A gel in the primary body cavity explains structures that occur in all of these five larval forms: (1) concave body surfaces bounded by thin epithelia and (2) muscles unopposed by other muscles. A gel filled primary body cavity invalidates deductions of morphogenetic mechanisms that assume a fluid filled cavity, an assumption implicit in many models of blastulation, gastrulation, and movement of mesenchyme cells. A gelatinous primary body cavity permits body plans and morphogenetic processes not possible with a fluid filled cavity and permits development of large larvae with little cellular material. The taxonomic distribution of gel filled body cavities is not known, but gel filled cavities are possible wherever fluid motion has not been demonstrated or is not a functional necessity.

Introduction

Many small animals lack both a muscular body wall and any apparent skeletal support, yet they maintain and alter elaborate body shapes. The embryos and larvae of echinoderms and hemichordates are examples of such animals, and the means by which they maintain their shapes are examined here. The means are not obvious. The body wall in these larvae is a thin epidermis. Some of these larvae (echinoplutei, ophioplutei) have a skeleton of calcite rods that support projecting arms (Fig. 1D), but others (tornaria, auricularia, bipinnaria) do not (Figs. 1ABC, 2AD). Even in the larvae with calcite rods, the epidermis is not everywhere stretched on the skeletal framework. In all of these larvae the thin sheet of epidermal cells appears inadequate to support itself. Two hypotheses for support of the epidermis have been stated or implied throughout an extensive literature on these larval forms. The most common assumption has been that the primary body cavity is filled with fluid and the thin body wall presumably supported by higher internal pressure (a hydrostatic skeleton), but no evidence for a hydrostatic skeleton has been presented. The other hypothesis is that the primary body cavity contains gelatinous supporting material (Ruppert and Balser, 1986; Summers *et al.*, 1987), perhaps like the mesogloea of jellyfish. Material that might have a supporting role has been demonstrated in the blastocoel of blastulae and gastrulae of echinoderms (Monné and Slautterback, 1950; Endo and Noda, 1977; Katow and Solursh, 1979; Kawabe *et al.*, 1981; Abed and Crawford, 1986; Summers *et al.*, 1987). However, these studies employed histological methods or electron microscopy. None of these studies tested the fluid or gelatinous character of the contents of the primary body cavity; and gelatinous material has not been obtained from live specimens. Thus, even a qualitative indication of the mechanical properties of material in the primary body cavity has been lacking.

Gelatinous material in the primary body cavity is here demonstrated to occur in all five types of feeding larvae of echinoderms and hemichordates, even in those with calcite skeletal rods. Available evidence therefore points to gelatinous support of these larval bodies. Support by a gel in the primary body cavity explains many features of shape, musculature, movement, and morphogenesis of these larvae.

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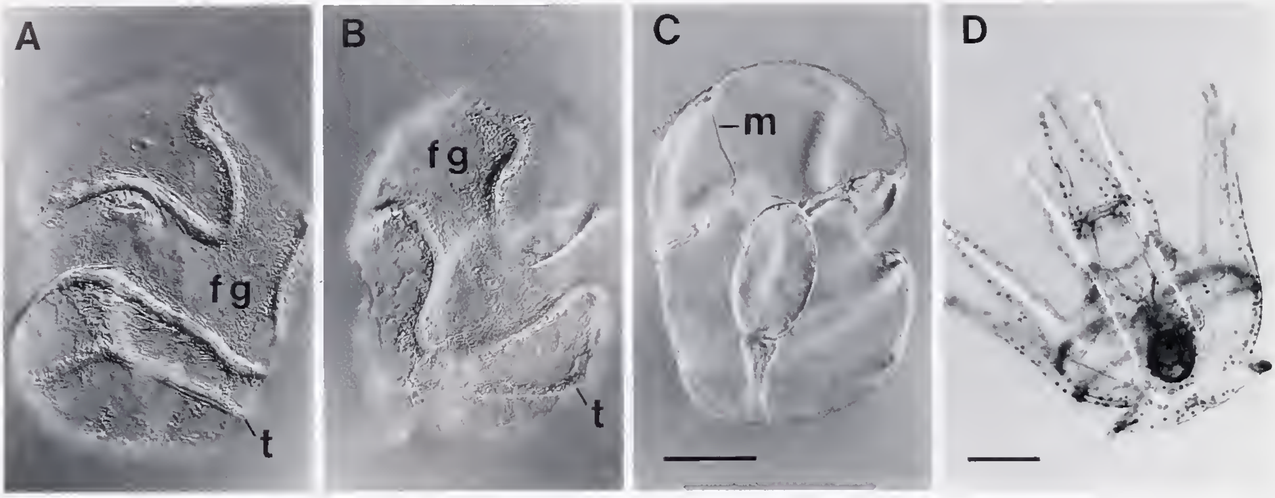

Figure 1. (A, B, C) Tornaria of *Ptychodera flava* with a concave to flat food groove (fg) upstream from the ciliated band. Body surfaces downstream from the band are flat to convex. Downstream areas include a separate locomotory band, the telotroch (t). The optical sagital section in C shows the thin epidermis, spaceous primary body cavity, and the muscle (m) between coelom and apical organ. Differential interference contrast optics. D: Echinopluteus of *Strongylocentrotus franciscanus* with skeletal rods in all arms and a less spaceous primary body cavity. Some skeletal rods are brightly illuminated because of partially crossed polarizing filters. Scale lines are 200 μm.

Materials and Methods

Species are listed in Table 1. Echinoderm larvae were reared at the Friday Harbor Laboratories from fertilized eggs in large (2 l) cultures (Strathmann, 1987). Ova and sperm of the hemichordate were obtained by leaving ripe adult worms in sand in still water at air temperature with occasional water changes until they spawned (Hadfield, 1975, and pers. comm.). After fertilization the hemichordate embryos and larvae were reared at the Kewalo Marine Laboratory in beakers with an airlift circulation (Miller and Hadfield, 1986). A single and unidentified large auricularia with wheel ossicles, obtained from the plankton near Honolulu, was also tested.

Live larvae were torn into two or more fragments with tungsten needles that were sharpened in molten $NaNO_2$ (T. E. Schroeder, pers. comm.). The larvae were dissected and observed in a suspension of particles of sumi ink ground on an ink stone (Schroeder, 1980a, b). The ink particles served several purposes. Because the ground substance of the primary body cavity itself was not visible with bright field or differential interference contrast (DIC) optics, the ink particles were needed to mark the boundaries of gelatinous material by decorating its surface and by exclusion of the ink suspension. The ink particles indicated motion of fluid, and the ink suspension mixed with body fluids. Because cells out of focus can resemble homogeneous and transparent gelatinous material, the ink particles were also useful as a marker for surfaces in focus.

Most larvae were dissected in seawater. Some specimens were dissected in isosmotic $MgSO_4$ or $MgCl_2$, which relaxed the larvae and may have aided removal of epidermis. However, because changes in concentration of calcium and other ions affect the viscosity of connective tissues of echinoderms (Motokawa, 1984; Wilkie, 1984), replacement of seawater with isosmotic $MgSO_4$ or $MgCl_2$ may have affected viscosity of materials in the primary body cavity. Except where isosmotic $MgSO_4$ or $MgCl_2$ is specified, seawater was the solution used in the results reported here.

I examined the fragments under a coverglass that was supported at its corners by pieces of plasticene (Clayola®). The test for gel was conservative in that gel was recorded only if a number of criteria were met. More than half the dissections demonstrated gel, and it is likely that gel was present even when these criteria were not met. Clear material from larval fragments was judged to be fluid if it sheared and eventually mixed with the ink suspension when the coverglass was depressed vertically and slid horizontally so as to compress or shear the specimen. Clear material unbounded by epithelia or membranous sheets was judged to be gelatinous if it did not mix with the ink suspension and if it returned to its original shape and position even when the coverglass was not returned to its original position. (Because of low Reynolds numbers, return of fluid to its original position after reversal of motion of the coverglass was expected and disregarded in these tests.) The presence or absence of bounding epithelia or other membranes was determined by observation under a 40× objective with DIC optics.

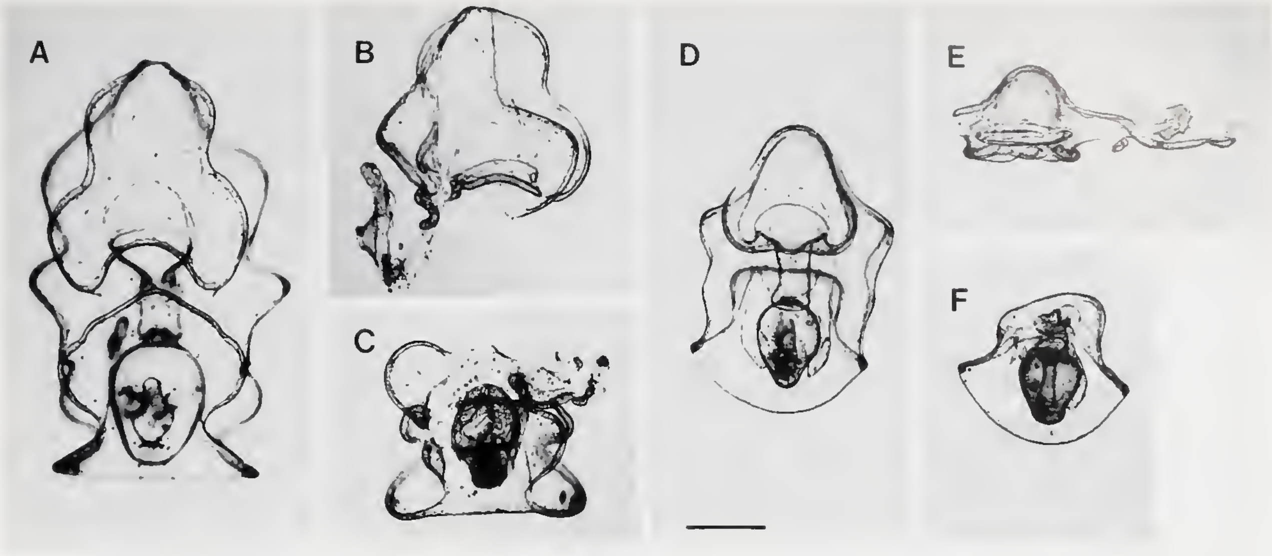

Figure 2. (A) Intact auricularia of *Parastichopus californicus* and anterior (B) and posterior (C) halves in seawater. (D) Intact bipinnaria of *Asterina miniata* with anterior (E) and posterior (F) halves of the same larva in isosmotic $MgCl_2$. The larvae were torn apart with needles but the halves retain their shape. Scale line is 200 μm for all figures.

When positive tests for gelatinous material were initially doubtful, tests were repeated until several specimens gave clear results. In addition, movement of ink, fluid, and gel was videorecorded for the tornaria so that impressions could be verified or rejected by repeated replay of events. Demonstration of the gelatinous material was not possible for every individual larva because ink adhered to the gelatinous material, accumulating on its surface and hiding it. Also, observation of gelatinous material depended on optical sections through a free surface, but orientation of larval fragments suggested that gelatinous material adhered to the glass slide and coverglass, thereby reducing opportunities to view torn edges of gel projecting into the ink suspension.

Results

Larvae of all five classes (Table I) had gelatinous material in their primary body cavities. The primary criteria for this conclusion were that the material excluded a suspension of ink particles during repeated compression and shear, did not shear like the surrounding fluid, returned to its original shape when deformed, and was not bounded by a membrane visible with a 40× objective and differential interference contrast (DIC) optics. These criteria were met by material from torn fragments of all five larval forms. The species tested in this way were *Ptychodera flava, Parastichopus californicus,* an unidentified large auricularia with wheel ossicles, *Asterina miniata, Ophiopholis aculeata,* and *Strongylocentrotus franciscanus.* All were dissected live, and all were in seawater, except for *A. miniata* and *O. aculeata,* which were in isosmotic $MgSO_4$. Ink particles were in brownian motion immediately adjacent to the surface of the gelatinous material. The boundary was distinct and abrupt. The surface of the gel in torn fragments was often irregular, as in Figure 3ABCD, not smooth as would be expected of an immiscible fluid or fluid bounded by a thin membrane. In most cases the material was visible at the edge of a torn fragment of the larva, as in Figure 3ABCEF. In a few cases (*P. flava, P. californicus, A. miniata, S. franciscanus*) I obtained isolated fragments of gel with only a few cells attached, as in Figure 3D.

In all five types of larvae the gel appeared transparent and uniform when viewed with a 40× objective and DIC optics (Figs. 3EF, and 4AB). The gel was not visibly granular, membranous, or fibrous, unlike cells, cell debris, and some other materials associated with cut fragments.

Some observations suggested that the gel filled most or all of the primary body cavity. In one dissection the cut

Table I

Larvae tested for gel in the primary body cavity

Phylum	Class	Species	Larval form
Hemichordata	Enteropneusta	*Ptychodera flava*	Tornaria
Echinodermata	Holothuroidea	*Parastichopus californicus*	Auricularia
	Asteroidea	*Asterina miniata*	Bipinnaria
	Ophiuroidea	*Ophiopholis aculeata*	Ophiopluteus
	Echinoidea	*Strongylocentrotus franciscanus*	Echinopluteus

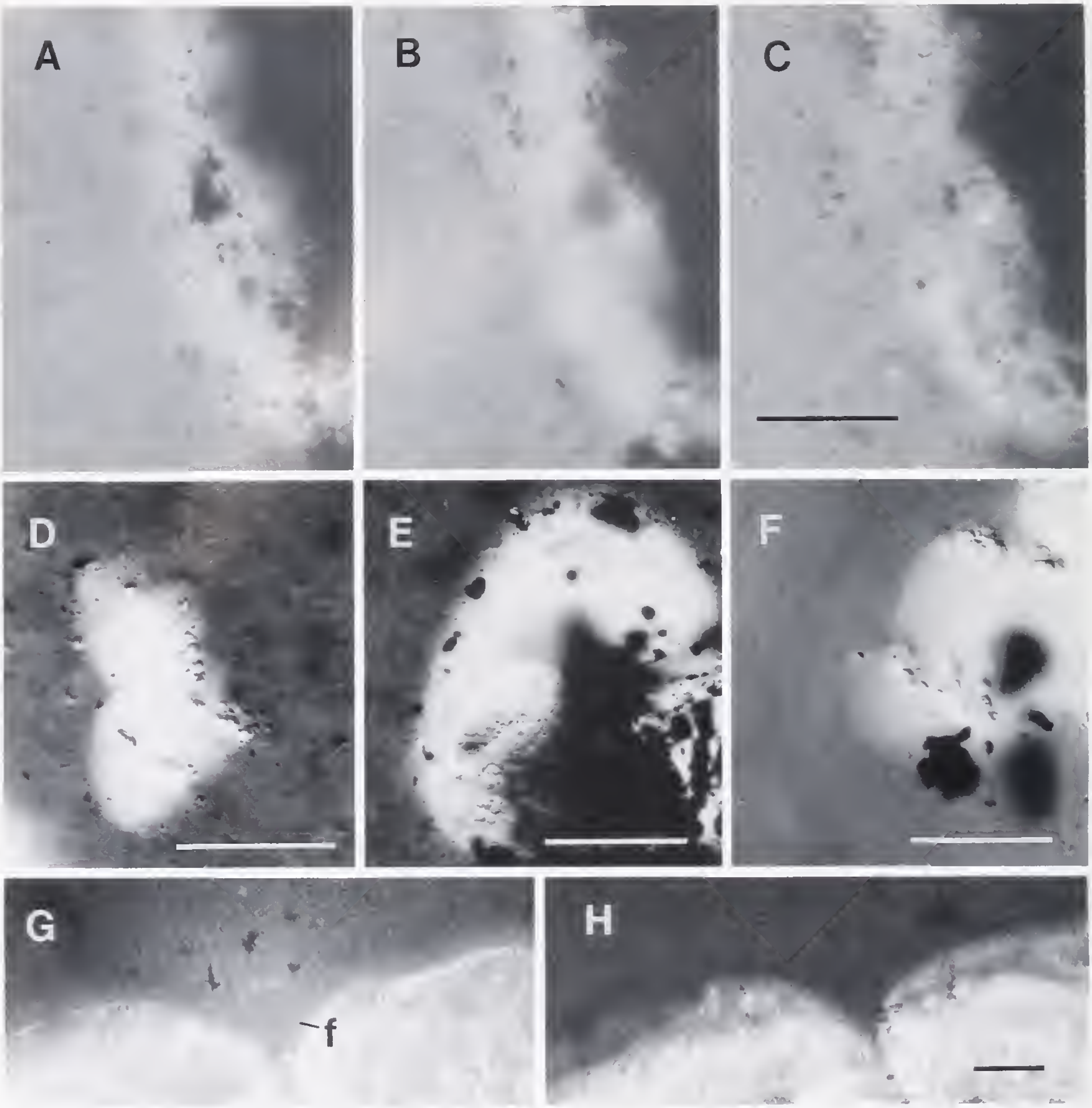

Figure 3. (A, B, C) High, middle, and low focus optical sections through gelatinous material at the cut edge of a tornaria of *Ptychodera flava*. Ink particles suspended in seawater show boundaries of the gelatinous material. (D) An isolated fragment of gelatinous material from cut fragments of the auricularia of *Parastichopus californicus*. The material is slightly compressed by the coverglass, surrounded by ink particles suspended in seawater, and partially bounded by ciliated cells on the right side. (E, F) Gelatinous material from pieces of the bipinnaria of *Asterina miniata* surrounded by ink particles suspended in isosmotic $MgSO_4$. Both pieces are strongly compressed by the coverglass. In E the gelatinous material surrounds part of the larval gut. In F the ink particles are in motion past the gelatinous material. (G, H) Piece of the tornaria of *P. flava* strongly compressed by the coverglass and surrounded by ink particles suspended in seawater. A clear fluid (f) has been extruded from the larval body in G but has been dispersed by motion of the coverglass in H. All scale lines are 50 μm.

half of a tornaria (*P. flava*) adhered to the coverglass. Pockets of ink suspension were trapped between this larval half and the coverglass, but excluded from the spacious primary body cavity to the side and below. The pockets of ink suspension were irregular and not bounded by a visible membrane. The ink particles were

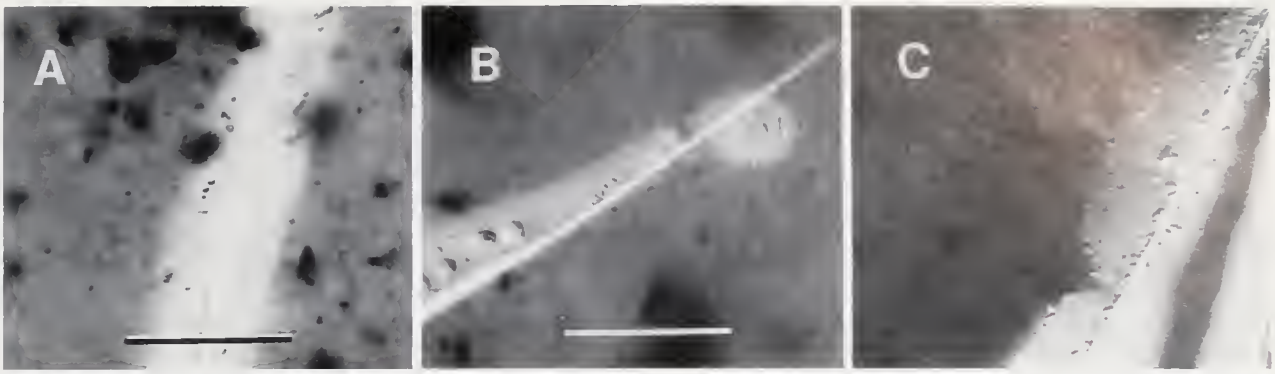

Figure 4. (A, B) Arms of the ophiopluteus of *Ophiopholis aculeata* with most of the epidermis stripped away; ink particles are suspended in isosmotic $MgSO_4$. A blob of gelatinous material is almost detached in B. Scale lines are 50 μm. (C) Arm of the echinopluteus of *Strongylocentrotus franciscanus* with most of the epidermis stripped away; ink particles are in seawater. C is at lower magnification than A and B. All specimens are strongly compressed by the coverglass.

in brownian motion immediately adjacent to the invisible surface of the bounding gel. I therefore concluded that the irregular pockets were the torn surface of a gel that filled the body cavity.

Gelatinous material also extended into arms of plutei from which the epidermis had been stripped (*O. aculeata* in isosmotic $MgSO_4$, *S. franciscanus* in seawater; Fig. 4). In one dissection of *S. franciscanus* an isolated blob of gel remained skewered on an arm rod like a kebab. Thus even body parts with skeletal support also had gelatinous support. The posterior part of the bodies of the ophiopluteus and echinopluteus also contained gelatinous material (same two species).

The gel partially or completely surrounded the gut (Fig. 3E) and esophagus of fragments of the bipinnaria of *A. miniata* (dissected in isosmotic $MgSO_4$).

There was also evidence against the alternative hypothesis that hydrostatic pressure maintains the larval shape. When tornariae (*P. flava*) and auriculariae (*P. californicus* and large larva with wheel ossicles) were torn in half in seawater, the thin epidermis did not collapse like a punctured balloon. The same result was obtained with the bipinnaria of *A. miniata* in both isosmotic $MgCl_2$ and seawater. Fragments of all three larval forms maintained the form that they had in the intact larva (as in Fig. 2 for auricularia and bipinnaria). Also, anterior and posterior halves of auriculariae (*P. californicus*) and bipinnariae (*A. miniata*) were distorted by compression under the coverglass but returned to their original shape when the coverglass was raised. Cut edges of the halves were not visibly sealed by epidermis; the primary body cavity appeared to remain open. Ink particles entered the oral cavity of the larval fragments but not the primary body cavity at the cut edges.

Fluid as well as gel was extruded from fragments of larvae under compression (Fig. 3GH). It is possible that parts of the primary body cavity were fluid but also possible that the fluid came from the gut or coelom or that some gel became fluid as a result of the dissection. I could not quantify comparisons, but it appeared that more fluid was extruded from the bipinnaria of *A. miniata* than from the auricularia of *P. californicus* when both were in seawater, and it was more difficult to demonstrate gel in the body cavity of *A. miniata* than of *P. californicus*.

The apparent volume of gelatinous material decreased when larval fragments were repeatedly compressed under a coverglass. The gelatinous material may have disappeared because it coated the glass, was coated with ink particles, or eventually became fluid when freed of the surrounding epithelia.

Discussion

Observations on living material demonstrate a gelatinous material in the primary body cavity of larval echinoderms and hemichordates. Support by gelatinous material in the primary body cavity explains many aspects of shape and musculature of these larvae and has implications for their development, as discussed below.

A gel can be any shape, and a gel filled body cavity may permit close approximations to optimal shapes. Placement of ciliary bands on ridges permits more effective propulsion of water (Emlet, 1985) and capture of particles (Strathmann, 1975). Depressed food grooves may aid retention of transported particles. In echinoderm and hemichordate larvae, ciliary bands are on ridges and much of the area for transport of food (upstream from the ciliated band) is concave (Figs. 1ABC, 2AD). These concave regions are bounded by a very thin and flexible epidermis. Such a shape is inconsistent with the inflated appearance expected with a hydrostatic skeleton. An inflated sac (with a hydrostatic skeleton) would either impose a smoother surface topography or require many additional internal cables or structures in the body wall to maintain the body's shape. The gel in the primary body cavity may also support the lobes or tentacles of some bipinnariae, auriculariae, and tornariae. Even some plutei, as in cidaroid echinoids (Emlet, 1988), have pronounced lobes that lack support from skeletal rods or other visible structures.

Support from gelatinous material permits development of large larvae with relatively little cellular material. The larval body is primarily a feeding machine, whose maximum capacity for clearing particles from suspension depends on the length of the ciliated band (Strathmann, 1971; M. Hart, pers. comm.). Strong positive allometry in ciliated band relative to body length and near isometry in band length to body protein and band length to metabolic capacity has been determined for echinoid larvae (McEdward, 1984, 1986). A strong positive allometry in band length to body length has been estimated for hemichordate larvae (Strathmann and Bonar, 1976). Except for the ciliated band, the body wall consists of an extremely thin sheet of epidermal cells. The body wall need not and does not provide much mechanical support for the ciliated band.

There is also an economy in muscles used for ingestion (circular esophageal muscles), rejection of particles (oral dilators of plutei and dorsal contractors of bipinnariae), and change of direction of swimming (dorsal contractors of bipinnariae) (Strathmann, 1971). None of these muscles is opposed by another muscle, yet the body rebounds to its resting shape when these muscles relax. The muscle that depresses the apex of the tornaria (Fig. 1C) also consists of a thin strand with no opposing muscle. For all of these activities, the gelatinous material in the primary body cavity is the only known elastic element that could oppose the muscles. Opposing muscles are unnecessary, and operation of muscles imposes few constraints on larval shape. Maintaining and changing shape requires very little cellular material in the body wall.

A gelatinous supporting skeleton also has implications for morphogenesis. The epithelia around the primary body cavity are not surrounding a fluid. An extracellular network of organic material in the primary body cavity of echinoderm embryos has been indicated by histological preparations and electron microscopy of preserved blastulae and gastrulae (Monné and Slautterback, 1950; Endo and Noda, 1977; Katow and Solursh, 1979; Kawabe *et al.*, 1981; Abed and Crawford, 1986). The extent of this material was difficult to ascertain because of shrinkage from fixation and preparation before observation. Berg and Akin (1971) collected fluid from echinoid blastulae by centrifugation. More recently Summers *et al.* (1987) demonstrated an extensive and oriented blastocoelic organic matrix in echinoid blastulae and gastrulae prepared by freeze-substitution. My observations with sumi ink extend those of Summers *et al.* (1987) by giving a direct indication of the mechanical properties of gelatinous material from body cavities of larvae. The observations also extend evidence of such material to additional classes of echinoderms and to hemichordates.

These observations on gel filled primary body cavities invalidate a critical assumption underlying a number of morphogenetic models that concern changes in shape of sheets of cells or movements of mesenchyme. A common practice in studies of the mechanics of morphogenesis from Gustafson and Wolpert (1962, 1963) to the present has been to deduce forces operating on sheets of cells from changes in the shape of tissues, but such models have implicitly assumed that blastocoels or other body cavities were fluid filled. This assumption is false for the primary body cavity in larval echinoderms and hemichordates and could be false for any body cavity in which fluid motion has not been observed. Also, mesenchyme cells are not traversing a fluid filled space in these larvae. The observations of morphogenetic movements remain sound, but interpretations of forces must be reexamined.

The role in morphogenesis of fibrillar material observed on the blastocoel wall in fixed blastulae and gastrulae may also need reinterpretation. Some of this material may be condensed gel.

A gel filled cavity could permit morphogenetic processes not possible in a fluid filled cavity. If epithelial or mesenchyme cells can dissolve and reform the gel, the gel filled cavity should permit greater freedom in generating forms and distributing cells than would a fluid filled cavity. The viscosity of connective tissue of adult echinoderms is under nervous control (Motokawa, 1984; Wilkie, 1984), and alteration of viscosity in the primary body cavity is a possibility that should be explored. Also, the mesenchyme cells in the primary body cavity of echinoderms and hemichordates are supported by a gelatinous material, and their movements may include complete detachment from other cells.

Gel filled cavities could play a major role in other embryos, larvae, and small adults. The plutei demonstrate that an internal mineral skeleton does not preclude the existence and usefulness of a gel filled body cavity. The taxonomic distribution of gelatinous support cannot be deduced from the presence or absence of other supporting structures.

Acknowledgments

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Temporal Changes in a Natural Population of Copepods

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Abstract. Adaptation was measured in a natural population of the estuarine copepod, *Eurytemora affinis* (Poppe). Significant changes occurred between generations in tolerance to elevated temperature and in body size, in both sexes, and in reproductive traits in females. If these changes were genetic, they did not result in different heritabilities for the traits and genetic variation was maintained in the population. However, genetic correlations between temperature tolerance and brood size in females showed significant changes between generations, lending support to two models for the maintenance of genetic variance. Linkage disequilibrium is discussed as an alternative explanation for the observed genetic changes that took place and for decreased hatchability of broods. Evidence for decreased hatchability, measured as proportion hatch, suggests summer diapause in this species.

Introduction

> "How much of the acclimatisation of species to any peculiar climate is due to mere habit, and how much to the natural selection of varieties having different innate constitutions, and how much to both means combined, is an obscure question."—Charles Darwin (1872)

Species can adapt to environmental variation in space or time physiologically, genetically, or by both means (Dobzhansky and Wallace, 1953). Physiological adaptation is accomplished at the individual level independent of available genetic variation. In contrast, genetic adaptation at the population level is dependent on available genetic variation. The degree to which a species uses either or both of these adaptive mechanisms is as obscure today as it was over one hundred years ago.

Chesapeake Bay populations of the estuarine calanoid copepod *Eurytemora affinis* (Poppe) are exposed to thermal conditions that vary from 0° to 30°C annually. Individuals experience only a portion of this thermal range since their generation time is relatively short (Ketzner and Bradley, 1982). Nevertheless, adaptation to ambient thermal conditions is likely to occur at both the individual and population levels.

A short-term assay of temperature tolerance in *E. affinis* has been used to predict long-term survival in increased temperatures (Bradley, 1976). Sexual dimorphism in this temperature tolerance trait has been observed repeatedly (Bradley, 1978a, b, 1982). Males expressed more additive genetic variance and occasionally less physiological tolerance for elevated temperatures than did females. However, physiological flexibility, measured as the difference between tolerances before and after acclimation to temperatures higher than those of standard culture regimes, was greater in females.

Genetic adaptation seems to be relatively less important to this species since individuals can survive and reproduce throughout the entire range of thermal conditions to which they are exposed. In spite of its physiological range, *E. affinis* did respond genetically with a sufficient rate of thermal change indicating that variation is both present and usable (Ketzner and Bradley, 1982). The question to resolve is why this species maintains genetic variation (Slobodkin and Rapoport, 1974).

There is no evidence for either dominance genetic variance or genotype by environment interaction in temperature tolerance in *Eurytemora* (Bradley, 1978a, b, 1986). Thus, selection models based on either temporal or spatial environmental variation (Gillespie, 1974; Haldane and Jayakar, 1963; Levene, 1953), or on a combination of the two (Ewing, 1979), are unlikely to account for the variation (Bradley, 1982). Similarly, heterozygote superiority appears unlikely since dominance is neces-

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sary for this phenomenon. Other models are more difficult to disprove. Density-dependent selection is probably absent since it would require a form of heterosis to maintain genetic variation. Frequency-dependent selection cannot be seriously considered without examining linkage relationships among many individual genes. Finally, stabilizing selection, even without heterosis, cannot be ruled out if mutation rates are high (Lande, 1975), which seems unlikely in this species.

Cyclical (seasonal) selection seems an obvious explanation for maintenance of genetic variation (Bradley, 1982, 1986). However, there was no consistent pattern among seasons for temperature tolerance among progeny taken from wild females and raised at 15°C in the laboratory. Neither was there a consistent pattern for seasonal progeny tested contemporaneously; parents from different seasons were maintained at 4°C (at which temperature generation time is about 4 months) until all collections were made and their progeny isolated and reared simultaneously at 15°C prior to testing (Bradley, unpub.). There is actually some indirect evidence against cyclical selection. Tolerances to high and low temperatures appear to be the same trait within both sexes, or at least are not negatively related (Bradley, 1976, 1986). Second, genotype by environment interaction for temperature tolerance, another potential indicator of seasonal selection, seems to be absent (Bradley, 1982, 1986).

Two models of selection to account for the maintenance of genetic variation in these copepods are still under serious consideration. One model is disruptive selection between the sexes (Mather, 1955), in which *E. affinis* females are selected for physiological flexibility and males for absolute temperature tolerance. Some evidence exists for the required genetic variation for flexibility (Ketzner, 1979). There is also some evidence for a negative correlation between absolute temperature tolerance and flexibility, and for differential selection between the sexes (Bradley, 1982). Under this model, genetic variation for both traits is preserved if selection is for a different trait in each sex.

An alternative model, genetic homeostasis (Lerner, 1954), states that genetic variation is maintained because selection for temperature tolerance is resisted by natural selection for fitness. This model requires genetic variation for temperature tolerance and for some other component of fitness, and a negative genetic correlation between the two traits. Temperature affects egg production, growth rate, size, and longevity in *E. affinis* (Heinle, 1970; Katona, 1971) and such traits may well undergo natural selection, allowing the possibility of genetic homeostasis. There is also some evidence for seasonal divergence in viability in *Eurytemora* (Bradley, 1982).

The issue in both the disruptive selection and homeostasis models is the sign and magnitude of the genetic correlations. The present study was designed to determine genetic correlations between temperature tolerance and other traits influenced by temperature or related to fitness of *E. affinis*.

Materials and Methods

The calanoid copepod *Eurytemora affinis* (Poppe) (Arthropoda, Crustacea) has peak densities during late winter and early spring in Chesapeake Bay beginning when water temperatures approach 5°C. It can be found throughout the year and is, therefore, exposed to a wide range of ambient temperatures (approximately 0–30°C). The actively swimming population is greatly reduced in summer and early fall.

Two samples (March and May, 1984) of *E. affinis* representing populations at early and late peak densities were taken from Bear Creek (39° 15′ latitude, 79° 27′ longitude, approximately). Temperatures *in situ* were 5.0 and 18.0°C, respectively. Ambient temperatures measured during previous years during the same periods also differed by more than 10°C. Based on generation times at various temperatures (see Katona, 1971), we assumed that adults present in the samples represented two distinct generations. We believe that predation by fish larvae reduced any generational overlap.

Ovigerous females were isolated into 50 ml of filtered bay water in 125-ml Erlenmeyer flasks within a few days of sampling. Each parent was removed when its egg sac was shed. Therefore the progeny in each flask were sibs. A 4:1 mixture of two algal species—*Monochrysis lutheri* (supplied by Howard Seliger's lab at Johns Hopkins University) and *Nitzchia* sp. (isolated from Bear Creek)—in log phase growth was added, as necessary, to maintain food concentrations near 10^5 cells per ml. Each parent was removed when its egg sac was shed. Progeny were raised in the same flasks under standard growth conditions (15°C, 14/10 L/D cycle, moderate light intensity) until ovigerous females appeared, whereupon members of both sexes from each sib group were isolated for testing.

All water used in the study came from Bear Creek and was stored at 4°C in 20-l polypropylene carboys prior to use. The treatment of bay water for algal food stocks differed from that used for copepod cultures in that algal water was charcoal-stripped to remove organic material (2 g powdered, HCl-washed, activated charcoal per liter stirred for 1 h), filtered through a .45-μm Millipore filter (HAWP 047-000) preceded by a Millipore cellulose prefilter (AP25-035-00) and autoclaved for 20 min at 121°C, 15 psi. The finished product stood at least 24 h to permit equilibration with atmospheric gases prior to use. Water for copepod culture was only filtered, as above, with the exception of water used for preliminary and reproduction studies. Early studies indicated no significant differences between the two water types when used for copepod cultures.

Filtered bay water was dispensed in 500-ml portions into 1-l Erlenmeyer flasks with 38 mm Ka-put tops (for algal cultures) or 1-l portions into 2-l flasks with screw caps (for general use). Plastic, disposable petri dishes were used as covers on the flasks to minimize evaporation yet allow sufficient air circulation. Sterile disposable 60 × 15 mm petri dishes (Falcon No. 1007) with 5-ml portions of treated bay water plus 5 ml food were used in many preliminary and reproduction studies.

Nutrient enrichments were added to the algal cultures. ESI_{S_1} enrichment (Provasoli, 1968) was added at 2%. A second enrichment, a modification of a B-vitamin mix formulated by Lewis (1967), was filter-sterilized and added at 0.5%. Adsorption of the vitamins in the B-vitamins mix onto algae promoted the growth and fertility of copepods better than direct addition of the vitamins to copepod cultures. Detailed culture methods are provided in Tepper (1986).

Parental and F_1 brood sizes were measured by isolating ovigerous females on petri dishes and counting under a dissecting microscope (25× magnification) only eggs within the brood sac. Ovigerous females were isolated in petri dishes with water and food (as above) or in vials. In the former case, proportion hatch (PH) of first brood was calculated as the number of hatched eggs divided by the brood size. Proportion hatch data were arcsin transformed prior to analysis. Survival after hatching of individuals in laboratory culture was near 100% and 70–80% of parental females released viable broods.

Heat tolerance (HT) was measured with a short-term nondestructive assay which correlates well with survival in elevated temperatures (Bradley, 1976). A modified version of the assay was used in this study consisting of immersing animals contained in individual vials into a computer-controlled water bath initially set at 32°C and raising the temperature 0.5°C every 5 min. HT was measured as the time taken for animals to succumb, that is to fail to respond to light shaking of the vials. Vials were prepared from Pyrex tubing, 21 mm ID by 100 mm high, which was fire polished and fitted with Nytex mesh (74 μm) on the bottom end. A run (one assay) consisted of a group of 16 vials, held 2 deep in a plexiglass rack, immersed simultaneously into the test tank (standard 10 gal aquarium) containing 5 ppt Instant Ocean made with distilled water. Each run contained four sib groups of four copepods each. Generally only families with at least four individuals of at least one sex were included in the analysis. A few broods with only three individuals of a sex were included. Sexes were tested separately. Thus the average tested brood size was around eight.

Protosomal (body) width (BW) was measured after the tolerance assays using a Zeiss compound microscope fitted with an ocular micrometer under 100× magnification. All measurements were taken on animals arranged dorsal-side up on depression slides. When immediate measurement was not possible, animals were stored at 4°C to arrest growth.

Seasonal genetic adaptation was suspected in previous studies (Bradley, 1982) and preliminary analysis indicated a difference between March and May samples in 1984. To further explore the possibility of temporal genetic adaptation, the principal comparisons made were between samples within sex. Statistical analyses included: means of sample family means and their variances, full-sib heritabilities, and genetic correlations based on family means. Student *t*-tests were used for within sex, between sample phenotypic comparisons of family means for all traits.

Full-sib estimates of heritability (h^2) were derived by partitioning the among-group mean square of a nested analysis of variance of families within tolerance runs into variance components (Falconer, 1981; Robertson, 1959a). Nesting was necessary to remove variation due to the physiological (HT) assay. An unbiased estimator of "average" or adjusted family size was used to calculate the maternal variance component (Brownlee, 1965). The exact variance of an h^2 estimate was derived from the variance of the intraclass correlation coefficient (t) among families within runs between phenotype and genotype (Fisher, 1941).

Genetic correlations (r_A's) between temperature tolerance and other traits were calculated using family means for each trait. Means tend to be normally distributed irrespective of the underlying distribution of the individual-within-family data (Kleinbaum and Kupper, 1978). Genetic correlations were calculated from a nested analysis of covariance based on family means. The covariance between a pair of traits was divided by the square root of the product of variances for each of the two traits. These estimates define the extreme limits of the genetic correlations in the same way that heritability estimates from full-sib analysis are upper estimates (Mode and Robinson, 1959). The standard error of each genetic correlation was calculated according to Robertson (1959b).

Two sets of data for female reproductive traits were available. First brood size was measured on the same individuals used for width and temperature tolerance measurements. A separate set of first brood sizes, as well as the proportional hatch of these broods, was measured on a second set of female sibs from the same families. The measurements of brood size were distinguished from one another as "MB1" and "RB1," respectively.

Results

The results of F_1 phenotypic comparisons between the two samples for the various traits are summarized in Ta-

Table I

Comparisons of first filial phenotypic means (of family means) and full-sib heritabilities from March and May 1984 sampling periods

Sex	Trait	Sample	Inds.[a]	Fams.[b]	Mean	S.D.	t	h^2	S.D.	df[c]	t
M	High temp. tol.	March	154	41	12.26[d]	2.35	**	.32	.19	30	n.s.
		May	187	50	8.53[d]	2.81		.72	.19	36	
F	High temp. tol.	March	163	41	13.82[d]	3.35	**	.90	.19	30	n.s.
		May	208	53	7.85[d]	2.33		.74	.18	38	
M	Width	March	155	41	326.04[e]	18.24	**	1.18	.17	30	n.s.
		May	183	50	309.78[e]	14.74		1.09	.17	36	
F	Width	March	162	41	443.84[e]	23.41	**	1.22	.17	30	n.s.
		May	209	53	421.65[e]	29.49		.99	.17	38	
F	M-brood size	March	164	41	44.14[f]	12.83	**	.91	.19	30	n.s.
		May	212	53	31.58[f]	12.02		1.16	.15	38	
F	R-brood size[g]	March	164	41	36.76[f]	11.73	**	1.07	.18	30	n.s.
		May	209	53	30.00[f]	11.57		1.41[i]	.12	38	
F	Proportion hatch[g]	March	164	41	.35[h]	.26	**	1.30	.15	30	n.s.
		May	209	53	.05[h]	.14		1.48[i]	.11	38	

[a] Individuals among families tested, [b]number of families tested, [c]number of families less number of tolerance runs (within sample), [d]minutes (time to succumb), [e]micrometers (converted from ocular units), [f]egg number, [g]represents a separate set of sibs from the same families, [h]proportion of hatched eggs (arcsin transformed prior to analysis), [i]significantly greater than 1.00 at $P < .05$, ** = $P < .01$, n.s. = $P > .05$.

ble 1. Temperature tolerance and size decreased significantly ($P < .01$) in both sexes between March and May samples. Length was also measured, but is not reported because no differences between length and width were found. Both measures of brood size and of proportion hatch decreased significantly ($P < .01$) in females during the same period.

In general, temperature tolerance increased proportionally with increasing ambient temperature due, in part, to acclimation (Bradley, 1978a). The temperature tolerance of wild-caught females, many of whom produced the progeny used in full-sib analyses, increased between the sample periods ($t = 2.63$, $P < .01$, $n_1 = 39$, $n_2 = 94$). Consequently, the decrease in temperature tolerance among F_1s between samples was puzzling initially. Physiological adaptation to laboratory thermal conditions (15°C) may have resulted in long-term acclimation by progeny from March in an upward direction and from May in the opposite direction. The opposing directions of acclimation by progeny would account for the discrepancy in these results. Parents had been in the laboratory two days before they were tested. Consequently, the results for wild-caught females represent conservative estimates of increased HT, relatively unbiased by laboratory acclimation, since complete acclimation requires additional time (Bradley, 1978a).

Decreased F_1 brood size between samples is consistent with previous results (Bradley, 1982; Katona, 1971). Decreased proportion hatch (PH) between samples is consistent with a previously found decrease of egg-to-adult viability for individuals grown under various temperature regimes in laboratory cultures (Bradley, 1982). Some of this variation may be maternal.

Estimates of F_1 heritabilities are also summarized in Table 1. Neither sex showed significant changes in heritability estimates between March and May samples for any of the traits studied. The estimates were surprisingly high.

The maximum theoretical value for heritability is 1. Many of the estimates exceed this and, for two of the traits, RB1 brood size (second set) and proportion hatch in the May sample, the 95% confidence intervals do not include the maximum theoretical value. Possible causes of biases in heritability estimates are discussed later.

The results of pairwise F_1 genetic correlations (r_A) calculated between temperature tolerance and the other traits are summarized in Table II. The genetic correlations were calculated from family means for each trait.

Table II

Comparisons of first filial genetic correlations (based on family means) between temperature tolerance and other traits from March and May 1984 sampling periods

Sex	Trait	Sample	r_A	S.D.	N[a]	df[b]	Z[c]
M	Width	March	.04	.21	41	27	n.s.
		May	.06	.14	50	33	
F	Width	March	−.16	.12	41	27	n.s.
		May	−.29[d]	.13	53	35	
F	M-brood size	March	.18	.14	41	27	*
		May	−.35[d]	.11	53	35	
F	R-brood size[e]	March	.35[d]	.12	41	27	**
		May	−.37[d]	.09	53	35	
F	Hatch percent[e]	March	.02	.11	41	27	n.s.
		May	−.31[d]	.09	53	35	

[a] Number of families tested, [b] number of families minus runs minus 3 for [c] Z test scores (Kleinbaum and Kupper, 1978), [d] significantly different from 0 at $P < .05$, [e] represents a separate set of sibs from the same families used for other traits, * = $P < .05$, ** = $P < .01$, n.s. = $P > .05$.

They provide estimates of the limits of genic correlations (the contribution of additive genetic covariance to the phenotypic covariance) since some of them may be increased by dominance and common environmental effects. Five of the eight genetic correlations from females were significantly different from 0 ($P < .05$), suggesting either pleiotropy or linkage disequilibrium (nonrandom associations between the genes for the different traits, perhaps due to their being on the same chromosome).

No change in the genetic correlation between HT and width was evident in either sex. However, females did show a significant change, from positive to negative, in r_A estimates between HT and brood size (with MB1 $P < .05$, with RB1 $P < .01$). The r_A between HT and proportion hatch also changed, from positive to negative, but not significantly.

Discussion

Temporal changes in temperature tolerance

Individuals sampled from March and May were exposed to quite different environments in the field during their development from egg to adult. The March generation experienced thermal conditions that cycled from 5° to near 0° and back to 5°C. In contrast, the May generation was exposed to increasing temperatures (from 5° to 18°C). The phenotypic differences observed in family means of laboratory-reared progeny suggest genetic changes occurred in the natural population, probably in response to parental exposure to field variation in thermal conditions. However, adaptation was not accompanied by a reduction in heritability (h^2) in either sex.

Heritability estimates for HT from male progeny were similar to previous findings (Bradley, 1978a, b, 1982). In general, female estimates were four times as large as previously found. No previous estimates of heritability for any of the other traits studied were available for direct comparison. Estimates of h^2 are often biased upwards when full-sib analyses are used due to (a) dominance (V_D) or common environment (V_{Ec}) components of variance and (b) large standard errors due, in part, to environmental differences under which selection (*in situ*) and testing (*in vitro*) take place (Bohren, 1975). However, in previous studies on the temperature tolerance trait either dominance (Bradley, 1978b) or maternal effects (Bradley, 1978a) were found.

Rearing conditions can produce a dominance-like effect (Istock, 1981) by presenting genotypes with novel environments for which prior genetic adaptation has not occurred. Laboratory procedures for the present study differed from earlier experiments in food type, quality, and concentration. Consequently, female heritabilities that were higher than previous estimates, might be inflated by V_D. Estimates of h^2 have varied with thermal or salinity rearing conditions, although not significantly (Bradley, 1986). Similar nonsignificant differences among heritability estimates due to small differences in thermal rearing conditions (10°, 12.5°, and 15°C) were found for *Eurytemora herdmani* (McLaren, 1976). In the present study, males should have had similarly inflated heritabilities over previous estimates, since temperature tolerance is apparently the same trait in both sexes (Bradley, 1978a); however, male estimates were somewhat lower than expected.

Previous results suggested that seasonal genetic diversity for HT in *E. affinis* was small or nonexistent (Bradley, 1982). The conclusion was supported by the observation that both heat and cold tolerance were either influenced by the same set of genes (*i.e.*, tolerance to both thermal extremes is the same trait) or at least were not negatively related (Bradley, 1976, 1978a, 1986). The same appears to be true of a desert pupfish; those from more thermally variable habitats were more resistant to both high and low temperature stress than pupfish from thermally stable habitats (Hirshfield, *et al.*, 1980). Other supporting evidence comes from the lack of genotype by environment interaction, which would be expected with seasonal diversity (Bradley, 1982, 1986).

The absence of genetic response in HT between samples is also consistent with a laboratory selection study by Ketzner and Bradley (1982) in mass cultures of *E. affinis*. In the present study the rate of *in situ* thermal change represented an increase of 0.21° per day (1.49° per week). Response to a variable *in vitro* temperature regime was primarily genetic when the rate of cyclical temperature change (between 10° and 22°C) was rapid (1°C per day), but was primarily physiological when the temperature changed 1° per week.

Temporal changes in other traits

Mean body widths in each sex, brood sizes, and proportion hatch all decreased significantly from March to May, but heritabilities generally remained constant and high.

There was a decrease in mean proportion hatch (PH), although once again heritabilities remained constant between samples. The decrease in PH may represent summer diapause in this species. Johnson (1980) has reported resting eggs in *Eurytemora*. Evidence of diapause in this study comes from the following three observations of the data. First, proportion hatch data are surprisingly consistent within families. All females within a family tended to have either greater than .80 or less than .20 proportion hatch. Families with a mix of high and low PHs were rare (<5% of population), as were females with intermediate PH values. Second, population mean PHs significantly decreased from March to May, and from the previous December to March (Tepper, 1986), suggesting a systematic decline in PH culminating in summer reductions in *E. affinis* populations. It remains to be shown that shed unhatched eggs are viable and that some environmental cue stimulates their continued development. Third, h^2 estimates for PH, which were very high, showed no change between samples. If summer diapause does occur, then the response would be purely physiological within a given habitat. Experimental selection for a change in the trait based on an environmental cue would further substantiate the relationship between diapause and proportion hatch.

The temporal genetic changes reflect natural phenomena occurring during the span of one or possibly two generations. There is ample precedent for rapid response to selection from artificial breeding experiments (see reviews by Falconer, 1981; Wright, 1977) and from natural populations (see review by Wright, 1978). Hairston and Walton (1986) recently inferred natural selection due to predation in freshwater copepod populations. They observed a rapid directional shift in timing of diapause when fish predators were no longer present. Seasonal and annual differences due to natural selection have been recorded for copepods (Battaglia and Lazzaretto, 1967) and bird populations (Smith and Zach, 1979; Boag and Grant, 1981). The pitcher plant mosquito produces several generations a year and apparently undergoes continuous natural selection for shifting degrees of larval diapause (Istock, 1981).

Laboratory results are often viewed as unrepresentative of values in nature since heritabilities and genetic correlations are functions of the environment in which they are measured (Stearns, 1976; Via, 1984). The absolute magnitude of the estimated genetic parameters is less important than change from sample to sample. Genotype by environment (G × E) interactions affect laboratory estimates of genetic parameters proportional to the degree *in vitro* conditions fail to meet the biological requirements of the organism (Robertson, 1957). The laboratory conditions used may have been stressful to *E. affinis*, but they were stressful to both samples. In addition, the relative contribution to phenotypic variance of G × E interactions is generally small (Falconer, 1981) and several studies have failed to find G × E interactions in natural populations (*e.g.*, Boag and Grant, 1978; Bradley, 1982, 1986; Boag, 1983; Murphy *et al.*, 1983).

Interactions among traits

Given sufficient physiological flexibility to meet most or all environmental circumstances, genetic adaptation would seem unnecessary. An early prediction was that genetic adaptation would be less important in females than males since female *E. affinis* have greater physiological flexibility and less additive variance for temperature tolerance than do males (Bradley, 1978a, b, 1982). However, genic (additive) variation was maintained for temperature tolerance in *E. affinis*, suggesting either varying selection pressure on males or periodic resistance to selection for temperature tolerance due to negative relationships with other traits that are also under selection pressure. The estimates of heritability of progeny reared in the laboratory were similar between samples. However, genetic changes from March to May are suggested by the changes in means of laboratory-reared progeny in all the variables.

The genic relationship between temperature tolerance and reproductive traits varied between samples suggesting differential selection pressures among traits in *E. affinis*. The genetic correlations between temperature tolerance and brood sizes, and between temperature tolerance and hatch percent consistently changed sign from positive to negative (Table II). This is the expected pattern. If selection were for both traits, pleiotropic genes affecting both traits in the same direction relative to selection would be fixed. Thus only pleiotropic genes affecting traits in opposite directions would contribute to the genetic correlation, tending to make it negative.

It is also possible that female *E. affinis* are selected for intermediate levels of egg production and temperature tolerance, due to competing energy requirements. In males such compromise may be unnecessary if sperm production requires less energy.

Trade-offs are common among life history characters in females of other species (Primack, 1978; Rose and Charlesworth, 1981a; review: Stearns, 1976) and directional selection applied to one life-history trait often results in a correlated response in another, or in several others. Selection for decreased development time resulted in a decrease in percent diapause in *Wyeomyia smithii* (Istock *et al.*, 1976). Selection for early fecundity resulted in increased early egg production whereas selection for late fecundity resulted in the opposite and in increased longevity in *D. melanogaster* (Rose and Charlesworth, 1981b; Luckinbill *et al.*, 1984). In the same species, selection for postponed reproduction increased longevity and decreased mortality rates (Rose, 1982). Laboratory adaptation in mass cultures of *Gammarus lawrencianus* for increased survival, early maturity, and high fecundity produced increased life expectancy and growth rate (Doyle and Hunte, 1981).

There are also cases in which balances among traits were absent: *e.g., Gambusia affinis* (Trendall, 1982), *D. simulans* (Murphy *et al.*, 1983), and *Thamnophis elegans* (Arnold, 1971). These exceptions may represent an inappropriate choice of traits for a particular species or inadequate sample sizes. They may also reflect more versatile homeostatic mechanisms.

Linkage disequilibrium or pleiotropic response?

Genetic correlations persist if caused by pleiotropy, but not if they result from linkage (Hazel, 1943; Lande, 1980). Since the observed changes were short term, they may have been due either to extensive linkage caused by unusual meiosis or to pleiotropy.

Linkage, in general, is unimportant for complex traits since these traits tend to be controlled by many genes scattered over several chromosomes (Lande, 1979; Stearns, 1980; Wright, 1977). However, work on *Drosophila* heat-shock genes indicates linkage among the genes involved in synthesis of the smaller of such proteins. Moreover, heat resistance in *Drosophila* can be modified through selection (Morrison and Milkman, 1978; Pelham, 1982). *E. affinis* has a set of heat resistance genes, some common to *Drosophila* and other organisms, and some not (Bradley *et al.*, 1988). The linkage relationships among these genes in *Eurytemora* is not known. *Drosophila* has many fewer linkage groups than does *Eurytemora*, so we cannot immediately argue the importance of linkage.

The absence of crossing over during gametogenesis (achiasmata) can cause linkage disequilibrium (non-random associations of genes) since gene combinations present in the parent are transferred intact to its offspring. Achiasmate females are common among invertebrates and can include entire orders (*e.g.*, Lepidoptera) or suborders (*e.g.*, the dipteran Brachysera) or only individual species within higher taxonomic classifications (White, 1973). Both cyclopoid and harpacticoid copepods are achiasmate in females and seem to be chiasmate in males (Ar-Rushdi, 1963; Wyngaard and Chinnappa, 1982). Thus, the consequences of linkage may be present in copepods, even with many chromosomes.

The occurrence of chromosomal ring structures during meiosis results in non-random segregation of chromosomes (White, 1973) and, therefore, can also produce linkage disequilibrium involving much of the genome during oogenesis. Cytological studies are rare for calanoid copepods, but the related freshwater cyclopoid species, *Mesocyclops edax*, has ring structures among chromosomes (similar to those formed in *Oenothera*). These are frequently formed during female meiosis (Chinnappa and Victor, 1979). Similar rings are formed during female gametogenesis in three species of harpacticoid copepods belonging to the genus *Tigriopus* (Ar-Rushdi, 1963). In *M. edax*, the number (one or more) and types (number and kind of chromosomes included) of rings can vary within and between populations, although within individuals the number and types were constant (Wyngaard and Chinnappa, 1982). In some cases, all *M. edax* chromosomes ($2n = 14$) were involved in one ring, creating two linkage groups during meiosis; one for each sex in the offspring, females being the heterogametic sex.

Whether in *E. affinis* ring formations occur during oogenesis is not known. Nor is it known if females are achiasmatic and heterogametic. It is clear that if *E. affinis* is similar to *M. edax* and *Tigriopus* species, which is likely, then the observed results could easily be the product of disequilibrium. The number of chromosomes involved in ring formation may be related to ambient thermal conditions or some other environmental cue. Further research in the cytology of *E. affinis* is warranted.

Ring formation in *E. affinis* could help explain the observed decreases in proportion hatch measured in progeny. Recessive lethals in female parents linked in ring structures could be passed on to progeny intact. If females are the heterogametic sex, then female progeny could be lost due to sex-linked lethals. McLaren and Corkett (1978) reported an unusual shift in the sex ratio of a population of the copepod *Pseudocalanus*. If ring structures occur in both sexes during gametogenesis, then inviable offspring would be even more prevalent.

The alternative explanation invoking pleiotropy can be offered for the changes observed in genetic correlations. As we suggested earlier, a trade-off, influenced by thermal variation in ambient conditions, may exist between temperature tolerance and brood size in females. Under this hypothesis, seasons represent different micro-environments to which the sexes adapt independently, not unlike micro-adaptation by individuals within seasons (Van Valen, 1965). Cyclic seasonal selection should then reverse the observed genetic correlations between HT and brood size in females during fall and winter.

Both linkage and pleiotropy may be important in the relationships between temperature tolerance and the other traits. Strong directional selection in large populations can fix either pleiotropic or linkage groups and allow those genes with antagonistic effects to segregate (Lande, 1982). The change in genetic correlations between temperature tolerance and brood size from positive to negative may have been the product of antagonistic genes. Although most of the changes between sampling periods were physiological, the thermal difference between samples was sufficient to produce some genetic adaptation. Earlier studies with *Eurytemora* found that beyond certain rates and ranges of change in temperature, genetic adaptation occurred. Exposure of copepods to differential temperatures in cooling waters through a power plant was sufficient to elicit a genetic response in HT of males (LaBelle and Bradley, 1982). Genetic adaptation also occurred during *in vitro* selection with the same magnitude of temperature change (Ketzner and Bradley, 1982).

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Suspension Feeding in Oscillating Flow: The Effect of Colony Morphology and Flow Regime on Plankton Capture by the Hydroid *Obelia longissima*

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Abstract. The effect of flow regime on the ability of the hydroid *Obelia longissima* to capture plankton was examined in a laboratory flume and a wave tank. Feeding effectiveness—the proportion of the gastrozooids with tentacles successfully capturing food in a fixed amount of time—is significantly greater in oscillating flow than in unidirectional flow at the same average velocity and particle flux. Thus quantitative feeding studies in unidirectional flow may seriously underestimate feeding in the field if flow in the natural habitat is unsteady. Increasing colony bushiness (weight/colony length) decreases feeding effectiveness in uni-directional and low frequency oscillating flow, but not in high frequency oscillating flow. Longer colonies (0.083 m) show significantly lower feeding effectiveness than short (0.033 m) colonies. This decrease in feeding effectiveness with increasing colony length and bushiness is offset by a rapid increase in polyp number with increasing colony length.

Introduction

Suspension-feeding organisms include representatives of almost every phylum, and the extreme radiation of many groups (*e.g.*, bivalve molluscs) has often been attributed to the acquisition of this habit (Jørgensen, 1966). Indeed, in benthic marine habitats, suspension-feeders are among the most conspicuous organisms. Because of the extreme prevalence of this lifestyle and the fact that many suspension feeders such as copepods form crucial links in the food web, suspension feeding mechanisms have attracted the attention of numerous biologists (*e.g.*, see reviews by Jørgensen, 1966, 1975, 1983).

Early studies of suspension feeding primarily examined particle capture by "active suspension feeders" (organisms that generate their own feeding currents) in still water (Jørgensen, 1966, 1975, 1983). More recently, with the use of unidirectional recirculating flumes to provide a well-characterized ambient current, it became possible to study suspension feeding in the laboratory by organisms that do not generate their own feeding currents, so-called "passive" suspension feeders (*e.g.*, Leversee, 1976; Taghon *et al.*, 1980; LaBarbera, 1981; Okamura, 1984; Patterson, 1984). Do these laboratory flumes provide sufficiently "realistic" flow regimes to permit extrapolation of the results of laboratory feeding studies to the performance of organisms in the field? Clearly, the answer to this question depends on the natural habitat of the organism under investigation.

The dynamics of flow in shallow water are often dominated by wave action. Although flow beneath waves is typically orbital, the orbits are compressed and flow is predominantly bi-directional (oscillating) close to the substratum (Bascom, 1964). Thus many shallow water, benthic organisms live in an oscillating flow habitat. If food capture by sessile organisms is neither quantitatively nor qualitatively different in oscillating flow regimes than in unidirectional flow regimes, then unidirectional flumes would provide an adequate system in which to study suspension feeding by most sessile organisms. If this is not the case, however, it will be necessary to re-evaluate studies of suspension feeding with greater attention to the dynamics of the flow regime in which the organism lives.

Suspension feeding organisms range from being rather rigid organisms (*e.g.*, mussels and scleractinian corals that maintain a constant morphology regardless of flow regime) to being flexible organisms (*e.g.*, hydroids that are deformed by moving water and are reoriented when

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the flow direction changes). Flexibility must be important to a suspension feeder since deformation will affect flow (and consequently particle flux) around the organism (*e.g.*, if branches collapse together, flow slows through the colony). While there are a few studies of suspension feeding in flexible organisms (*e.g.*, Leversee, 1976; Okamura, 1984), these were made in uni-directional flow regimes, essentially steady-state situations. Holland *et al.* (1987) examine particle capture by the crinoid *Oligometra serripinna* in unsteady flow. The flow regime they used, however, provided brief periods (2 s) of reversed flow alternating with long (10 s) periods of flow in one direction. While this approximates some surge conditions, it is not a good approximation of fluid movement under waves. The purpose of this study is to measure particle capture by the hydroid *Obelia longissima*, a "representative" flexible organism, in uni-directional and oscillating flow regimes. This will permit evaluation of the importance of flow regime (unidirectional *vs.* oscillating) to suspension feeding organisms. I will also examine how flexibility may affect the performance of a suspension feeder.

Materials and Methods

Animal collection

Specimens of the thecate hydroid *Obelia longissima* were collected from the dock at Friday Harbor Laboratories, Friday Harbor, Washington. Colony selection from the field was randomized using a random number table. The animals were transferred to seawater tables where they were held no longer than 4 hours prior to their use in experiments.

Experimental design

A 3 by 2 factorial design was used to assess the effects of colony length and oscillation frequency on feeding by hydroid colonies. Feeding indices (defined below) were determined for colonies of 2 different lengths in each of 3 different flow regimes. The average length of short colonies was 0.033 m (S.D. = 0.005), while the average length of long colonies was 0.083 m (S.D. = 0.012). Flow regimes were: (1) uni-directional flow, (2) "low-frequency" oscillating flow at a frequency of 0.33 Hz with a peak velocity of 0.087 m/s, and (3) "high-frequency" oscillating flow at a frequency of 1.04 Hz with a peak velocity of 0.068 m/s. In all three flow regimes, the average (root mean square for oscillating flow) velocity was 0.025 m/s, comparable to that seen by these *Obelia longissima* in the field (Hunter, 1988). The high-frequency oscillation corresponds to wind-generated chop commonly experienced by *Obelia* in the field, while the low frequency oscillation was more like the waves generated by large boat (ferry) wakes (Hunter, 1988). Since the mean velocity and particle concentration was equivalent in all treatments, the mean number of particles moving past the colony per cross-sectional area of tank per unit time (particle flux) was also equivalent in all treatments.

Oscillating flow tank

Oscillating flow was produced using a wave tank (Hunter, 1988). The tank produced a uniform (less than 5% variation in velocity across the working section), smooth flow field. Oscillation frequency could be varied from a period of 4.0 s to 0.9 s at peak velocities ranging from 0.01 m/s to 0.25 m/s.

Unidirectional flow tank

Uni-directional flow was produced in a recirculating flow tank (Vogel and LaBarbera, 1978). The velocity profile across the tank was adjusted with baffles and the flow velocity did not vary by more than 7% of the mean velocity across the working section of the tank.

Food

Fresh plankton was used as the food source. Plankton was collected by towing a plankton net (80 μm mesh) alongside the pier at Friday Harbor Laboratories. The plankton was washed out of the net with seawater and then stained for 10 min with rhodamine B (0.005% in seawater). The stain solution was washed out of the plankton, using a 50 μm Nitex mesh to retain the plankton. The bulk of the remaining plankton ranged in size from 50 μm to 200 μm. It consisted of eggs, veligers, plutei, copepods, many larval crustaceans, and other types of invertebrate larvae.

The concentration of the stained "stock suspension" of living plankton was determined by counting a subsample (25 ml) in a Bugarov tray on a dissecting microscope. This permitted determination of the appropriate dilution for use in an experiment. The plankton concentration in the flow and wave tanks was adjusted to approximately 0.75 particles/ml immediately prior to each experiment.

The concentration of the same size range of plankton in the hydroid's habitat (adjacent to the pier) typically ranged from 0.05 to 0.9 (mean = 0.6, S.D. = 0.5, n = 10) particles/ml over the duration of this study, although there was a one-week period where the concentration of megalops larvae alone reached 1.05/ml. Thus the particle concentrations used in these experiments were comparable to those in the field, being slightly higher than mean field values. The experiments had to be kept short to prevent particle digestion before counting and thus it was necessary to use a slightly high particle concentration to assure a countable number of fed polyps in the small colonies.

The same stock suspension of plankton was used for each set of treatments on a given day. Thus, although the plankton composition changed through the season, the change was comparable across all treatments. The order of treatments was randomized each day to eliminate possible biases due to diel cycles of feeding activity. The treatment water was sampled immediately prior to and subsequent to the running of a feeding experiment. The particle concentrations in these samples were determined by counting as indicated above.

Measure of feeding effectiveness

To measure feeding effectiveness, every tentacle-bearing polyp in a colony was counted. Those polyps that took plankton were easily identified by the bright red color of the stained plankton contained within the coelenteron (gut). It was not possible to count individual plankton particles within a polyp, so it was assumed that all fed polyps consumed equivalent volumes of food. This was justified since, if the polyp fed at all, it was usually completely full (pers. obsv.). Typically, the fed polyp was bloated, completely filling the theca (the cup-like exoskeleton surrounding the polyp).

A feeding treatment lasted 15 min. Colonies fed for a longer time (up to 1 h) contained more fed polyps, indicating that the colonies were not satiated at the end of a 15-min feeding treatment.

Since larger colonies have more polyps, it was necessary to normalize the counts of fed polyps to compensate for simple size effects. Two normalizations were determined: (1) "polyp feeding effectiveness," a measure of how many polyps fed successfully, was defined as the fraction of polyps capable of feeding (gastrozooids fully differentiated and possessing intact tentacles) that actually captured food. (2) "Colony feeding effectiveness," a measure of feeding rate relative to the biomass supported by that food, was defined as the number of polyps that captured food divided by the mass of the colony.

Polyps were counted immediately after the experiment, so there was not enough time for digestion to alter the apparent number of fed polyps. As digestion proceeded (longer than 40 min), the stain moved down through the polyp into the common coelenteron (gut) of the colony. If the stain appeared below the level of the theca, the colony was not used.

Polyp feeding effectiveness is a ratio bounded between zero and one. All measures of polyp feeding effectiveness were arcsin transformed prior to statistical analysis (Sokal and Rohlf, 1969). Colony feeding effectiveness, however, may be greater than one, and was not transformed prior to analysis.

Effect of uni-directional flow velocity

Differences observed in feeding performance between uni-directional and oscillating flow might have been due to the fact that oscillating flows had higher peak velocities than did the uni-directional flows. To ascertain if increased flow velocity *per se* could produce higher feeding rates, feeding was measured at three velocities in uni-directional flow: 0.025 m/s, 0.050 m/s, and 0.10 m/s. The fast velocity (0.10 m/s) was greater than the peak flow velocity in any of the oscillating flow treatments. At a fixed particle concentration, however, faster flows provided a greater particle flux past the colony. To separate the effects of differing flux from those due simply to fluid velocity, two experiments were done. In the first experiment, the particle concentration was equivalent at each flow velocity. In the second experiment, particle concentration was adjusted to provide an equivalent particle flux at all three velocities.

Results

Oscillation frequency

Obelia colonies feeding in oscillating flow had a significantly higher polyp feeding effectiveness (# fed polyps/# polyps capable of feeding) than those colonies feeding in unidirectional flow at the same average particle flux (2-way ANOVA, $F_{(2,120)} = 22.7$, $P < 0.0001$, for flow effects) for both long and short colonies (Fig. 1). There was no significant difference in particle capture between colonies in the low-frequency and colonies in the high-frequency flow regimes (Student-Newman-Keuls post-hoc test, Damon and Harvey, 1987). This was true for both long and short colonies (2-way ANOVA, $F_{(2,120)} = 0.57$, $P = 0.57$, for interaction).

Similarly, colony feeding effectiveness (# polyps fed/colony dry weight) is greater in oscillating than in unidirectional flow (2-way ANOVA, $F_{(2,114)} = 5.58$, $P = 0.005$ for flow effects). Colony feeding effectiveness appeared directly proportional to oscillation frequency in long colonies. Short colonies had a higher colony feeding effectiveness in low-frequency oscillating flow than in unidirectional or high-frequency oscillating flow (Fig. 2). The interaction between flow regime and colony length was high, but not significant (2-way ANOVA, $F_{(2,114)} = 2.62$, $P = 0.08$ for interaction), suggesting that the difference in frequency dependence between long and short colonies was not significant.

Particle concentration

There were no significant differences in initial particle concentration (2-way ANOVA $F_{(3,120)} = 0.69$, $P = 0.56$) or particle depletion (2-way ANOVA $F_{(3,120)} = 0.91$, $P = 0.44$) during the experiment between any of the treat-

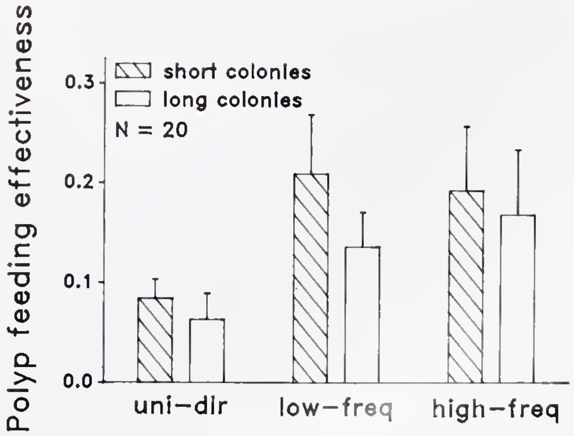

Figure 1. Polyp feeding effectiveness (number of fed polyps/number of polyps capable of feeding) in uni-directional flow, low frequency (0.33 Hz) oscillating flow, and high frequency (1.04 Hz) oscillating flow for long (0.083 m) and short (0.033 m) colonies. Error bars are 95% confidence intervals.

ments, indicating that the feeding effects were not due to differences in particle concentration between the treatments.

Colony length

Long colonies had significantly more fed polyps than short colonies (2-way ANOVA, $F_{(1,120)} = 34.7$, $P < 0.001$, for length). This simply reflects the fact that long colonies have more feeding polyps than short colonies. However, polyp feeding effectiveness (# fed polyps/# polyps capable of feeding) is actually less for long colonies than for short colonies (2-way ANOVA $F_{(1,120)} = 7.8$, $P = 0.006$ for length) (Fig. 1). In contrast, colony feeding effectiveness (# fed polyps/colony weight) is not significantly different for long and short colonies (2-way ANOVA, $F_{(1,114)} = 2.27$, $P = 0.14$ for length) (Fig. 2). This suggests that the increase in polyp number in larger colonies offsets the lowered polyp feeding effectiveness.

Colony bushiness

Bushiness was defined as the weight per unit length of colony. Polyp feeding effectiveness showed a slight decrease with increasing bushiness in unidirectional flow (regression, $F_{(1,38)} = 5.5$, $P = 0.02$, $R^2 = 0.13$) and low frequency oscillating flow (regression, $F_{(1,38)} = 6.3$, $P = 0.02$, $R^2 = 0.14$), but not high-frequency oscillating flow (regression, $F_{(1,38)} = 0.41$, $P = 0.52$, $R^2 = 0.01$) (Fig. 3).

Colony feeding effectiveness (feeding/weight) showed similar trends. There was a slight decrease in colony feeding effectiveness with increasing bushiness in uni-directional flow (regression, $F_{(1,35)} = 5.2$, $P = 0.028$, $R^2 = 0.13$) and low-frequency oscillating flow (regression, $F_{(1,36)} = 5.9$, $P = 0.019$, $R^2 = 0.14$), but not in high frequency oscillating flow (regression, $F_{(1,34)} = 3.3$, $P = 0.08$, $R^2 = 0.09$) (Fig. 4). Thus, it can be seen that while bushiness has a weak effect on feeding performance in uni-directional and low-frequency oscillating flow, high-frequency flow seems to mitigate its effect.

Unidirectional flow velocity

There was a significant increase in polyp feeding effectiveness as flow velocity (and consequently particle flux) increased (Kruskal-Wallis 1-way ANOVA, $\chi^2 = 11.42$, $P = 0.003$) (Fig. 5). When the particle concentration was altered to provide equivalent particle flux at all three velocities, there was no difference between any of the flow velocities (Kruskal-Wallis 1-Way ANOVA, $\chi^2 = 1.74$, $P = 0.42$). This suggests that the increase in polyp feeding effectiveness with increasing flow velocity was due to differences in particle availability (flux), not to differences in flow velocity *per se.*

Colony feeding effectiveness (# polyps fed/colony weight) showed a similar trend, with significant differences at equivalent particle concentration (Kruskal-Wallis 1-way ANOVA, $\chi^2 = 8.16$, $P = 0.017$) and no significant differences at equivalent particle flux (Kruskal-Wallis 1-way ANOVA, $\chi^2 = 1.67$, $P = 0.43$) (Fig. 5). Thus differences in colony feeding effectiveness are also due to differences in particle availability, not to differences in flow velocity.

Location within the colony

Polyp feeding effectiveness was not affected by the location (height) of the polyp within the colony. There was

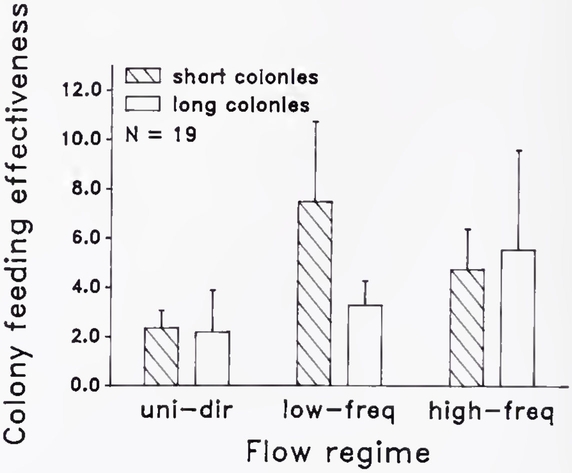

Figure 2. Colony feeding effectiveness (number of fed polyps/colony mass) in uni-directional flow, low frequency (0.33 hz) oscillating flow, and high frequency (1.04 hz) oscillating flow for long (0.083 m) and short (0.033 m) colonies. Error bars are 95% confidence intervals.

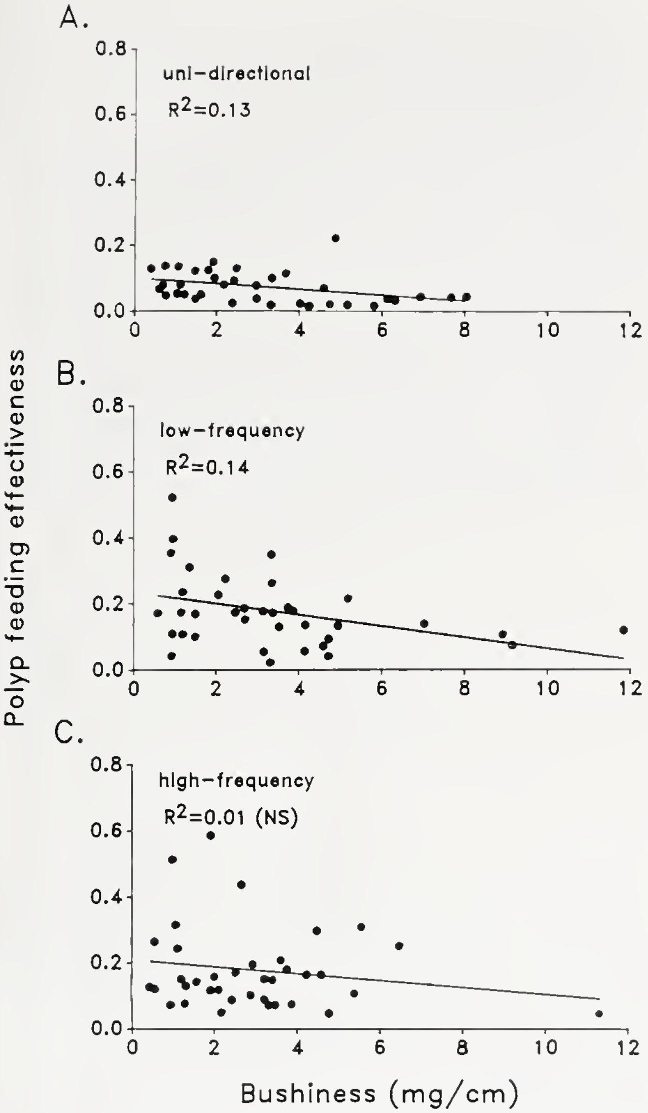

Figure 3. Polyp feeding effectiveness (number of fed polyps/number of polyps capable of feeding) plotted as a function of colony bushiness (colony weight/colony length) for uni-directional flow, low frequency oscillating flow (0.33 hz), and high frequency oscillating flow (1.04 hz).

no significant difference in polyp feeding effectiveness (# polyps fed/# capable of feeding) in polyps from the top or the bottom of the colony (Fig. 6) in any flow regime (nested ANOVA, $F_{(2,228)} = 0.16$, $P = 0.85$).

Discussion

Flow regime and feeding

Four factors strongly affect feeding by flexible organisms in oscillating flow regimes: (1) colony reorientation, (2) fluid resampling, (3) local particle depletion, and (4) reduced relative flow velocity. These factors interact in a complex fashion, making it difficult to predict *a priori* how flow regime will affect feeding. I will briefly discuss each of these effects.

Reorientation

In contrast to rigid organisms, flexible organisms are passively reoriented to changes in flow direction. In uni-directional flow, as the flow velocity increases, the organism tends to be pushed over towards the substratum. The faster the flow, the more the organism leans over. This positions more of the organism within the boundary layer (region of reduced flow velocity adjacent to the substratum), so the flow velocity, and consequently the particle flux, is reduced.

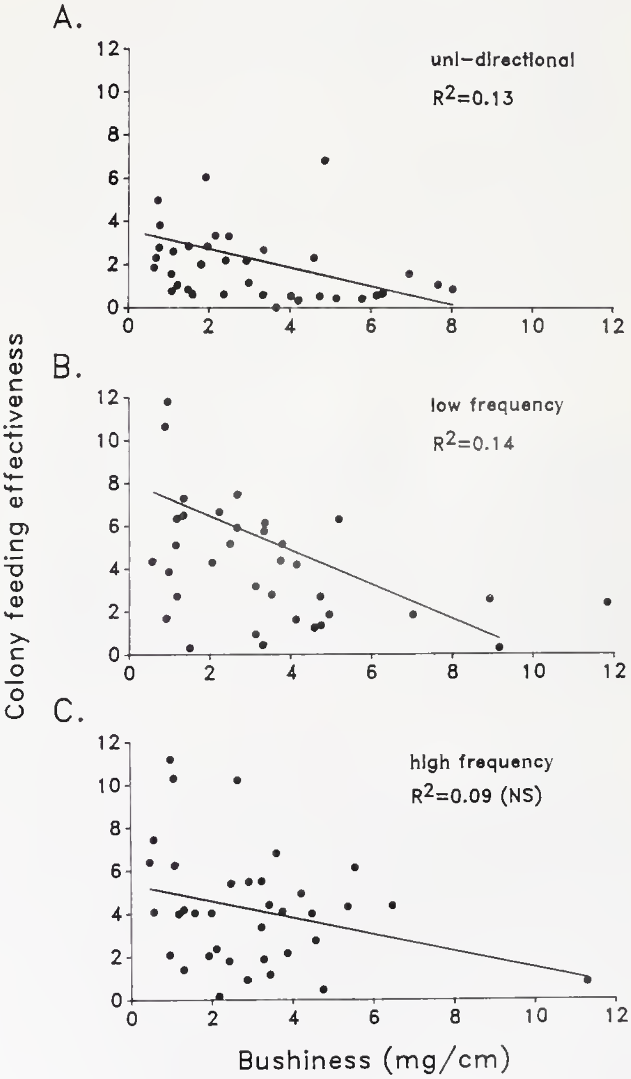

Figure 4. Colony feeding effectiveness (number of fed polyps/colony mass) as a function of colony bushiness (colony weight/colony length) for uni-directional flow, low frequency oscillating flow (0.33 hz), and high frequency oscillating flow (1.04 hz).

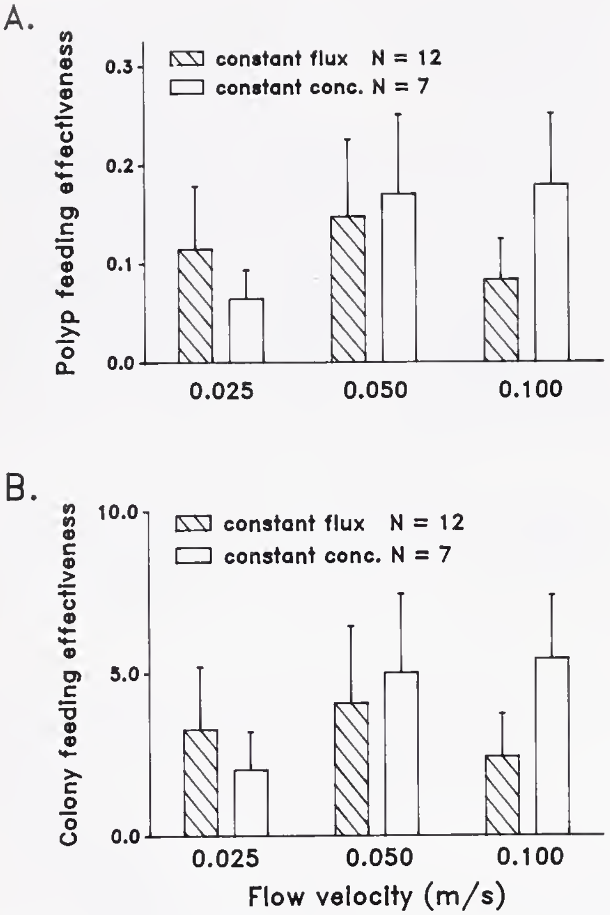

Figure 5. (A) Polyp feeding effectiveness (number of fed polyps/number of polyps capable of feeding) as a function of flow velocity in "slow" (0.025 m/s), "medium" (0.050 m/s), and "fast" (0.100 m/s) uni-directional flow. Data is plotted for experiments done at constant particle concentration (blank bars), in which the particle flux increases with flow velocity, and altered particle concentration (striped bars), where the particle flux is equivalent at all three flow velocities. All error bars are 95% confidence intervals. (B) Colony feeding effectiveness (number of fed polyps/colony mass) in the same uni-directional flow treatments.

Reorientation also reduces local flow velocity (and consequently particle flux) by "self shading." As the colony is pushed parallel to the flow direction, polyps downstream near the tip of the colony are "shielded" from the flow by the upstream polyps. The colony branches collapse together, and the water tends to pass around the entire colony rather than between the colony branches. Water velocity through the colony is thus reduced. This effect was clearly observed by injecting fluorescein upstream of the colony and timing the passage of the dye through the colony. Self shielding has been observed to reduce flow through numerous other benthic organisms. For example, Chamberlain and Graus (1975) described retarded flow through various scleractinian corals caused by "self shading." Okamura (1984) has invoked "shading" as a mechanism by which upstream colonies of the bryozoan *Bugula stolonifera* reduced feeding by downstream colonies.

In oscillating flow, these effects are mitigated. As oscillation frequency increases, the flow direction reverses before the colony has a chance to lean over. The colony tends to maintain an orientation normal to the flow direction and "self shading" is reduced. Thus, based simply on the reorientation effects, one would expect to see enhanced feeding in oscillatory flow relative to unidirectional flow. This prediction is supported by the data of this study and is consistent with the observation that the hydroid *Aglaophenia pluma* grows more rapidly in oscillating than in uni-directional flow (Svoboda, 1970). This enhanced growth may be mediated by higher particle (food) capture rates in oscillating flow.

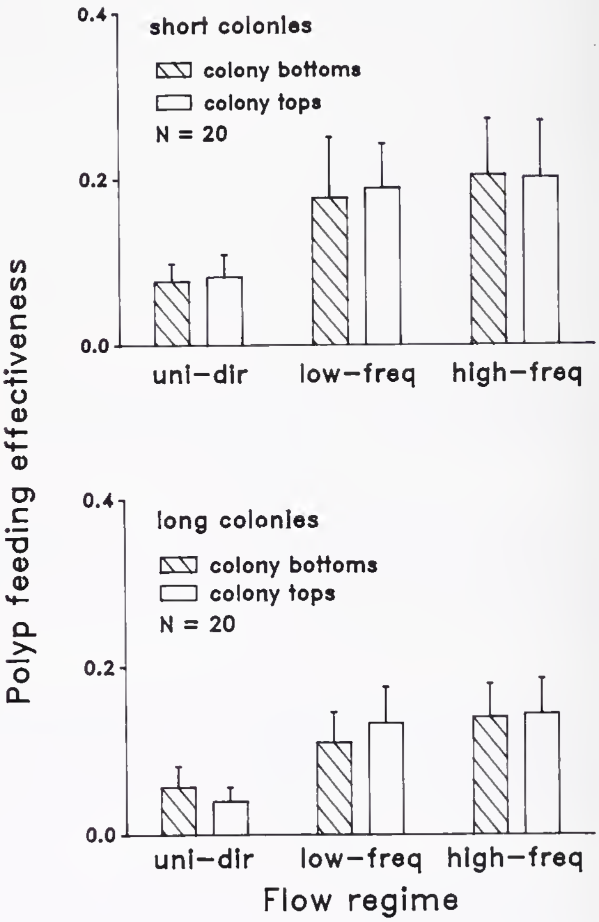

Figure 6. Polyp feeding effectiveness (number of fed polyps/number of polyps capable of feeding) in uni-directional flow, low frequency (0.33 hz) oscillating flow, and high frequency (1.04 hz) oscillating flow. Polyps from the top 25% (blank bars) and the bottom 25% (striped bars) of long (0.083 m) and short (0.033 m) colonies. Error bars are 95% confidence intervals.

Fluid resampling

In oscillating flow, the same "parcel" of water tends to move back and forth repetitively over the colony. The colony thus samples the same volume of water over and over again. The size of this "parcel," and thus the volume resampled, is determined by the amplitude of the oscillation. The amount of new water seen by the colony is a function of the rate of mixing of this parcel with fluid farther away from the colony and the rate of advection (*e.g.*, flow due to the tidal current onto which the waves are superimposed) of water past the colony. Dye blobs injected around colonies in the field show rather long residence times (1–2 min). Similar effects have been seen in the field around gorgonian sea fans in wave surge (Koehl, pers. com.). This is in dramatic contrast to uni-directional flow where water often remains only briefly around a colony before being swept downstream.

As the food particles within the resampled volume of fluid are consumed feeding effectiveness may decrease. Thus, by increasing local particle depletion, fluid resampling in oscillating flow may tend to reduce feeding effectiveness and thus acts to reduce the feeding "enhancement" effect of reorientation.

Local particle depletion

Local particle depletion may occur in oscillating flow as a consequence of fluid resampling. However, local depletion may occur in uni-directional flow even though there is no fluid resampling. In uni-directional flow, passive reorientation (leaning over) of the colony, has the effect of placing the low (basal) polyps in the colony upstream of the more distal polyps. Thus basal polyps may reduce the particle concentration of the water by the time it reaches the distal polyps. Similarly, increasing colony bushiness (or the presence of colonies upstream) may result in local particle depletion, much as the presence of colonies of the ectoproct *Bugula stolonifera* decreased feeding by downstream colonies possibly through local particle depletion (Okamura, 1984). Thus local depletion due to colony reorientation or increasing colony bushiness may reduce feeding effectiveness in uni-directional flow, while local depletion due to fluid resampling may reduce feeding effectiveness in oscillating flow.

The effect of local particle depletion on feeding effectiveness must depend on the rate of transport of particles into the depleted volume of fluid. In oscillating flow, the colony waves back and forth in an undulatory fashion. This caused the branches to "flail like oars" and appeared to increase mixing in the vicinity of the colony. Patterson (1984) has shown that increasing turbulence (in a uni-directional flume) results in more uniform distribution of particle capture throughout colonies of *Alcyonium siderium*, indicating that local mixing can be important to suspension feeders.

Dye blobs injected around the hydroids showed rapid mixing in the wave tank, but were quickly swept downstream away from the colonies in the uni-directional flume. Dye injected around hydroids in the field is rapidly mixed around the colonies, but *Obelia* in the field forms rather dense canopies and the dye is retained within the canopy. The data showing enhanced feeding in oscillating flow relative to uni-directional flow are consistent with the hypothesis that increased local mixing mitigates the effects of local particle depletion.

Reduced relative flow velocity

A reduction in flow velocity relative to the organism is a feature peculiar to flexible organisms in oscillating flow regimes. When the direction of flow reverses, there is a period of time during which the distal portion of the organism is moving *with the flow* as the colony reorients. During this reorientation, the flow velocity (and consequently, the particle flux) relative to the colony tip should be reduced or zero. When the colony has fully reoriented (*e.g.*, when it is completely "strung out" in the direction of flow) the distal region will again experience flow. Longer colonies require a larger amplitude of oscillation to be fully reoriented. For any given oscillation frequency and amplitude, there is a colony length at which a colony never achieves full reorientation before the oscillation reverses.

In light of the "reduced relative flow velocity" (and consequently reduced particle flux) at colony tips, polyp feeding effectiveness near the tips should be lower than polyp feeding effectiveness near colony bases. Further, this difference should be more pronounced in long as opposed to short colonies because long colonies show a greater reduction in relative velocity at the tip than do short colonies (Hunter, 1988). The data do not support this hypothesis. There is no significant difference in feeding effectiveness between the top 25% and the bottom 25% of the colony (Fig. 6).

It is possible that local mixing due to the "flailing" of the branches of long colonies maintains a high local particle concentration; so the reduced water velocities relative to the colony tips do not significantly reduce particle flux. Alternatively, particle retention may be increased at reduced relative velocity. At lower flow velocity particles are less likely to be swept out of the "polyp's grasp." Many authors (*e.g.*, Wainwright *et al.*, 1976; Okamura, 1984; Harvell and LaBarbera, 1986) suggest that particle capture and retention at "high" flow velocities might be a problem for suspension-feeding organisms. It is suggested that flexibility, by reducing "relative" flow velocities, also reduces variability in flow speed and thus facilitates suspension feeding (Wainwright *et al.*, 1976; Harvell and LaBarbera, 1986). Evaluation of these hypotheses will require actual visualization of particle capture.

Flow velocity and feeding

Feeding effectiveness is higher in oscillating than in uni-directional flow at the same average velocity. Yet, the peak velocity in oscillating flow is higher than in uni-directional flow (0.087 m/s *vs.* 0.025 m/s, respectively) and the difference in feeding may simply be due to this difference in peak velocities. Simply increasing flow velocity (from 0.025 m/s to 0.050 m/s) in uni-directional flow at constant particle concentration increases polyp and colony feeding effectiveness (Fig. 6). This is due to an increase in particle availability (flux past the colony). If particle flux is held constant, this increase in feeding with increasing flow velocity disappears (Fig. 6).

By measuring feeding both at constant flux and at constant particle concentration, it is possible to separate effects due to particle availability from effects due to flow velocity *per se.* Most studies have analyzed the effect of velocity on feeding without separating these two confounding effects (*e.g.*, Leversee, 1976; LaBarbera, 1981; Okamura, 1984).

Colony morphology

Increases in colony bushiness (colony weight/colony length) result in a small but significant decrease in both polyp feeding effectiveness (Fig. 4) and colony feeding effectiveness (Fig. 5). As colony bushiness increases, more gastrozooids (mouths) occupy the same volume. Thus, if the volume is well mixed, feeding effectiveness should increase. However, if the volume is not well mixed, and colony feeding results in a local particle depletion, then feeding effectiveness should decrease because of the lack of particles available for consumption. Increased local mixing, by stirring more particles into this depleted volume of water, would tend to restore the original particle concentration. If the effect of bushiness on feeding effectiveness is mediated through particle depletion, high frequency oscillating flow, by increasing mixing, should eliminate the dependence of feeding effectiveness on bushiness. (Dye blobs around the colony dissipate more rapidly in high frequency flow than in low frequency flow, suggesting that mixing is more rapid in the high frequency flow regime.) The data are consistent with this hypothesis (Figs. 3, 4). Clearly, as the dependence of feeding effectiveness on bushiness shows, the effect of changes in morphology on performance may depend on the flow regime. Increasing colony length also decreased feeding effectiveness (Fig. 2). This might be explained as a consequence of low relative flow velocities (and particle flux) near the tips of long colonies. However, there was no significant difference in polyp feeding effectiveness between colony tops and bottoms (Fig. 6). A strong correlation between length and colony bushiness (Fig. 7) may account for the length effect. Since increased bushiness decreased feeding effectiveness (Figs. 3, 4), and since increased length was associated with increased bushiness, then increased length should be associated with decreased feeding effectiveness. If this is so, one would expect to see the effect of length on feeding to be mitigated in high-frequency oscillating flow since bushiness had no effect on feeding in high frequency flow. This is not the case. Bushiness, however, increased rapidly with increasing length (Fig. 7). Long colonies may be so much more bushy than the short colonies that a local particle depletion occurred even in high-frequency oscillating flow.

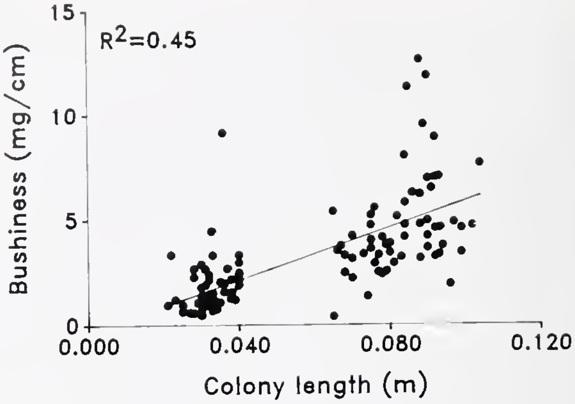

Figure 7. Colony bushiness (colony weight/colony length) as a function of colony length. Bushiness shows a significant increase with increasing colony length (regression, $F_{(1,117)} = 143$, $P < 0.001$, $R^2 = 0.45$).

Flow regime and performance

Clearly an examination of suspension feeding as a function simply of flow velocity may prove misleading if the organism normally lives in an unsteady flow habit. Fluid resampling and reduced relative velocity do not occur in uni-directional flow. The effects of reorientation and local particle depletion may vary greatly with flow regime. In addition, variations in morphology may have different effects on the rate of suspension feeding in different flow regimes. Further, the behavior of the organism may change with changing flow regime. For example, in oscillating flow, the encrusting bryozoan *Membranipora* tends to achieve an attitude with polyps far more distended than in uni-directional flow (pers. obs.). Such changes in behavior or performance with changing flow regime have been documented in many groups (*e.g.*, spionid polychaetes, Taghon *et al.*, 1980; bivalved molluscs, Walne, 1972; bryozoans, Okamura, 1987).

In summary, the flow habitat occupied by flexible organisms is not simply equivalent to the ambient flow regime. Rather, it is a complex interaction of colony morphology and water movement. Factors such as colony reorientation, fluid resampling, local particle depletion, and reduced relative flow velocity may strongly effect the performance of the organism. Further, reduced relative flow velocity and fluid resampling are factors present for flexible organisms in oscillating, but not in uni-directional flow regimes. This fundamental difference between oscillating and uni-directional flow regimes, suggests that it is inappropriate to estimate feeding performance of suspension-feeding organisms in uni-directional flumes if their normal habitat is subject to oscillating flow.

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Ultrasonic Biotelemetry of Muscle Activity from Free-Ranging Marine Animals: A New Method for Studying Foraging by Blue Crabs (*Callinectes sapidus*)

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Abstract. The design and utilization of a small (16 g in air, 6 g in water) ultrasonic transmitter for animal tracking and bio-telemetry of muscle activity are described. To our knowledge this is the first use of such devices to telemeter a specific behavior (ingestion) from a free-ranging marine predator. The transmitter produces regularly recurring short tracking pulses, and long pulses triggered by the action potentials of a muscle. Pulses are transmitted through conductive estuarine water by a piezoelectric ring transducer at a frequency of about 75 KHz. The preparation of a subject animal, insertion of electrodes, and attachment of the transmitter are described for the telemetry of mandibular muscle contraction in the blue crab. The transmitter provides a signal that corresponds unequivocally with feeding activity and allows enumeration of bites required to consume a food item. Number of bites, and feeding time, are both positively correlated with the size of the prey specimen. As a test of the technique's feasibility, a blue crab was equipped with one of the transmitters and released in a subestuary of Chesapeake Bay. The crab was tracked continuously for 96 hours while every contraction of the mandibular muscle was recorded. The crab traveled 4000 m along the subestuary at an average speed of 12 m/h, but showed periods of rapid movement of up to 325 m/h. The crab fed 2–7 times per day, with a feeding bout comprising 15–2750 bites. The limited data did not indicate that either movement or feeding exhibit a diel or tidal cycle.

Introduction

We describe the design of a new ultrasonic telemetry transmitter for relatively small marine or estuarine animals. In addition to producing a locating signal, it transmits a signal triggered by myopotentials occurring during muscle contraction. Thus, if activity of a muscle can be linked to a specific behavior, the transmitter can be used to obtain detailed records of that behavior from animals that cannot be directly observed. We use this device to telemeter contraction of the mandibular muscle of blue crabs (*Callinectes sapidus*) ranging freely in a subestuary of the Chesapeake Bay. Here we provide preliminary data on foraging activity, principally obtained from the first few crabs on which the method was tested. Subsequent papers will present the results of large-scale studies which have followed.

Ultrasonic tracking is a well-established tool in freshwater ecosystems, and is usually the only practical transmission mode in marine and estuarine waters which, being conductive, rapidly absorb radio transmissions. [Use of electromagnetic radiation in seawater is limited to very low frequencies and short distances, *e.g.*, Phillips *et al.*'s (1984) system for studying the movements of rock lobsters (*Panulirus cygnus*) near shore.]

Ultrasonic transmission has been used primarily to track a wide variety of fish, as well as cetaceans, pinnipeds, turtles, sea snakes, and alligators (Stasko, 1975; Stasko and Pincock, 1977). Several species of invertebrates also have been tracked with ultrasonic telemetry, including queen conchs (*Strombus gigas*) (Clifton *et al.*, 1970), American lobsters (*Homarus americanus*) (Lund and Lockwood, 1970), king crabs (*Paralithodes kamtschatica*) (Monan and Thorn, 1973), Norway lobsters (*Nephrops norvegicus*) (Chapman *et al.*, 1975), prawns (*Macrobrachium rosenbergii*) (Peebles, 1978), a portunid crab (*Scylla serrata*) (Hill, 1978), and spiny lob-

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sters (*Panulirus argus*) (summarized in Herrnkind, 1980). All of these studies provided data on movements of free-ranging animals, from which behavioral information (*e.g.*, habitat utilization) could be derived.

Ultrasonic telemetry can be used to provide directly more detailed behavioral information, in addition to location. Data on water temperature, depth, or swimming speed or direction have been telemetered from various vertebrates, providing such information as dive profiles and thermal preferences (*e.g.*, Nelson, 1978; Westerberg, 1984; Rubinoff *et al.*, 1986; review by Wilson *et al.*, 1986). In some studies, ultrasonic biotelemetry has been used to record physiological functions of free-ranging animals, particularly heart rate in large vertebrates (*e.g.*, Wardle and Kanwisher, 1974).

Bottoms and Marlow (1979) described the design of a small ultrasonic tag that uses changes in electrical impedance between two electrodes to transmit activity of a moving part, *e.g.*, heart rate or scaphognathite beat. However, their tag was only tested successfully in the laboratory, providing preliminary data for heartbeat in a crab (*Cancer pagurus*). Because their method uses continuous transmission, it requires large battery capacity and is not suitable for long-term monitoring of small animals in the field. Therefore we elected to telemeter behavioral data by pulse encoding, keeping the duty cycle of power-hungry output stages, and hence the size of the battery needed, to a minimum.

Blue crabs were chosen as the study animal in developing this system for several reasons. First, the crabs are dominant benthic predators regulating the abundance and species composition of epi- and infaunal communities in oyster bars, beds of submerged vegetation, and soft bottom habitats throughout much of their range (from Cape Cod to Brazil) (Virnstein, 1976; Holland *et al.*, 1980; Laughlin, 1982; Hines *et al.*, in prep.). Second, because this species forms the basis of a major commercial and recreational fishery along the east and Gulf coasts of North America, there is an extensive data base on its physiology and ecology (Millikin and Williams, 1984). Finally, we wished to develop a transmitter and technique of wide applicability. A system sufficiently sensitive, small, and robust to be used on estuarine blue crabs (15 cm carapace width) would also be suitable for a variety of other estuarine and marine species.

Materials and Methods

The ultrasonic transmitter (Fig. 1) produces regularly recurring tracking pulses, and long pulses triggered by the amplified action potentials of a muscle and lasting for the duration of the contraction. The pulses are radiated from a piezoelectric cylinder and transmitted through water as high-frequency (about 75 kHz) sound. Technical details of the electronic design are presented in Wolcott and Hines (1989), and detailed schematics and component lists are available from the first author; only a brief functional description of the circuit is given here.

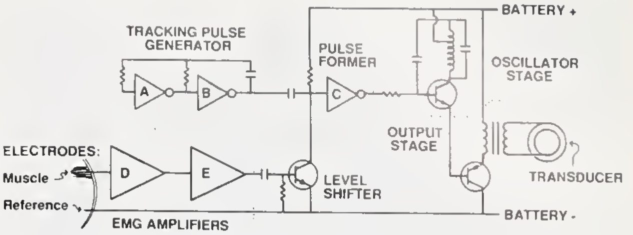

Figure 1. Schematic diagram of the telemetry transmitter. The triangles in the astable multivibrator (A, B) and pulse former (C) represent subunits of a CMOS logic circuit (4049 hex inverter). The EMG amplifiers (D, E) are MC1776 op amps configured as non-inverting amplifiers with ac voltage gains of 100.

The amplitude of the useable signal from the muscle of interest, the mandible closer, was estimated to be a few hundred microvolts. To determine the amount of voltage gain needed to switch the timing circuitry, prototype amplifiers were constructed with micro-power integrated operational amplifiers, and tested by connecting them to long wires from electrodes implanted in a crab (see below) in an aquarium. (A similar determination would have to be made each time the device is adapted to a different system). Two op-amp gain stages, amplifying the biopotentials a nominal 10,000 times, proved adequate when followed by a single-transistor amplifier that shifted the signal to logic levels. The logic level output serves as an input to the CMOS integrated circuit used for all timing of pulse signals.

The timing section of the circuit, an astable multivibrator and pulse former, produces a regular, 15 ms long "tracking pulse" at about 1 s intervals. A train of action potentials from a muscle forces the pulse former into the "on" state, producing a long, nearly continuous burst superimposed on the tracking pulses (see below). When "on," the timing circuit turns on a low power ultrasonic oscillator, which in turn drives a higher power output stage connected via a matching transformer to the piezoelectric ring transducer. The latter converts the 75 kHz electrical signal to acoustic (ultrasonic) vibration.

The transmitter was reduced to a printed circuit and assembled using surface-mount components. A lithium battery (CR-1/3-N, 160 mAh, 3 g), powered the transmitter for more than 2 months. Completed transmitters were encapsulated originally by coating with melted vinyl-modified paraffin wax (KK731, U. S. Wax). To reduce labor, later units were inserted into vinyl electrical sleeving (Alpha PVC-80-5/8″), the ends glued shut, and

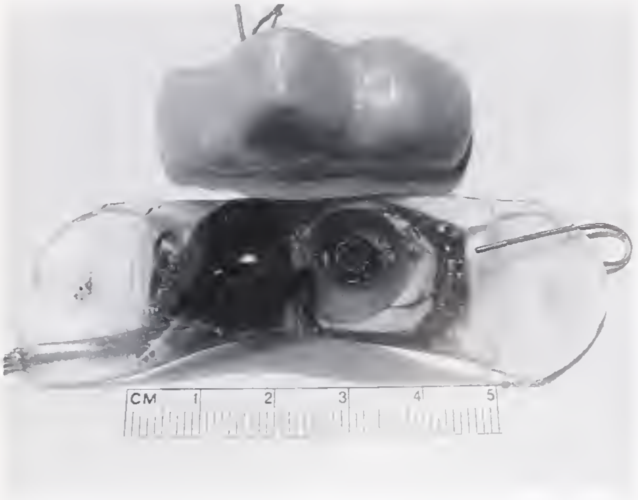

Figure 2. Completed transmitters. Above: encapsulated with wax. Below: encapsulated in vinyl sleeving. Hooked wire at right plugs the oil-fill hole.

the package backfilled with mineral oil until all air bubbles were eliminated (Fig. 2).

After some practice, we could prepare a crab, insert the electrodes, and attach the transmitter to the crab in about 20 min. We determined by dissections that, in the blue crab, the muscles that close the mandibles originate on the front of the exoskeleton, ventro-lateral to the eye sockets (Fig. 3), where electrodes could be inserted conveniently into the end of the muscle and immobilized by fastening to the exoskeleton. Reference electrodes were sited about 1 cm laterally to enter the hemocoel away from any potentially interfering muscles. The surface of the exoskeleton at the electrode sites was cleaned and dried by swabbing with acetone. Using a small ball-tipped dental bit (no. 1/2), holes were drilled nearly through the calcified layer of the exoskeleton at the desired locations, with care not to drill completely through the exoskeleton. A patch of thin rubber (*e.g.*, heavy dental dam) was glued to the area using a high-performance cyanoacrylate glue (Dexter Hysol 2-C-500) to provide a septum through which electrodes could be inserted without loss of blood. (Bleeding interferes with glue bonding in addition to causing undesirable physiological effects.)

Electrodes were fabricated of number 0 stainless steel insect pins cut into 1 cm lengths. The electrodes were bent so that they penetrated about 3 mm inside the exoskeleton. The active (muscle) electrode was angled to lie parallel to the muscle fibers when the outside portion was cemented flat to the exoskeleton. The reference electrode was bent about 90 degrees to enter the hemocoel perpendicular to the skeletal surface. The distal ends of the stainless steel electrodes were solder-tinned using phosphoric acid flux, thoroughly rinsed with tap water, and soldered to the transmitter's flexible leads of PVC-insulated no. 36 stranded copper wire. The electrode tips were then pushed through the rubber septum and the predrilled holes in the exoskeleton. The electrodes were immobilized and waterproofed by gluing a second layer of rubber over the entire area (Fig. 3).

The body of the transmitter, if wax-coated, was affixed to the crab with strips of thin rubber stretched over the tag and glued to the carapace. Transmitters encapsulated in vinyl sleeving were tied on with wire passed through holes at each end of the package and passed around the blue crab's large lateral spines (Fig. 3). Crabs were always placed in a large aquarium after tagging, to verify electrode placement and transmitter operation. Their behavior was observed until they fed and transmitted appropriate signals.

Results

Crabs appeared to adapt readily to carrying the transmitters. Instrumented intermolt crabs were held in the laboratory for as long as two months with no apparent ill effects, or tracked in the field for up to three weeks before being lost. Transmitters and attachments weighed about 15 g in air and 5.5 g in water when coated with wax, or 16 g and 6 g, respectively, when encapsulated in oil-filled vinyl. The weight in air is about 7.5% that of a 150 mm carapace width (point-to-point), intermolt blue crab. In water, the transmitters probably contribute drag and

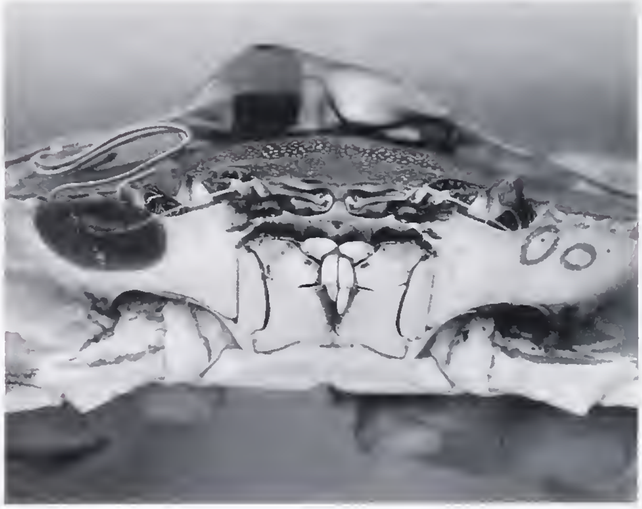

Figure 3. Crab fitted with transmitter, showing locations and typical electrode insertion. Right side of photograph: outlines on crab's "face" delineate origins of the mandible muscle. Predrilled hole for the active electrode is visible within the outline of the medial muscle origin; that for the reference electrode is lateral to the outer muscle origin. Left side of photograph: electrodes, inserted through rubber patch applied over predrilled holes, and sealed with a second rubber patch.

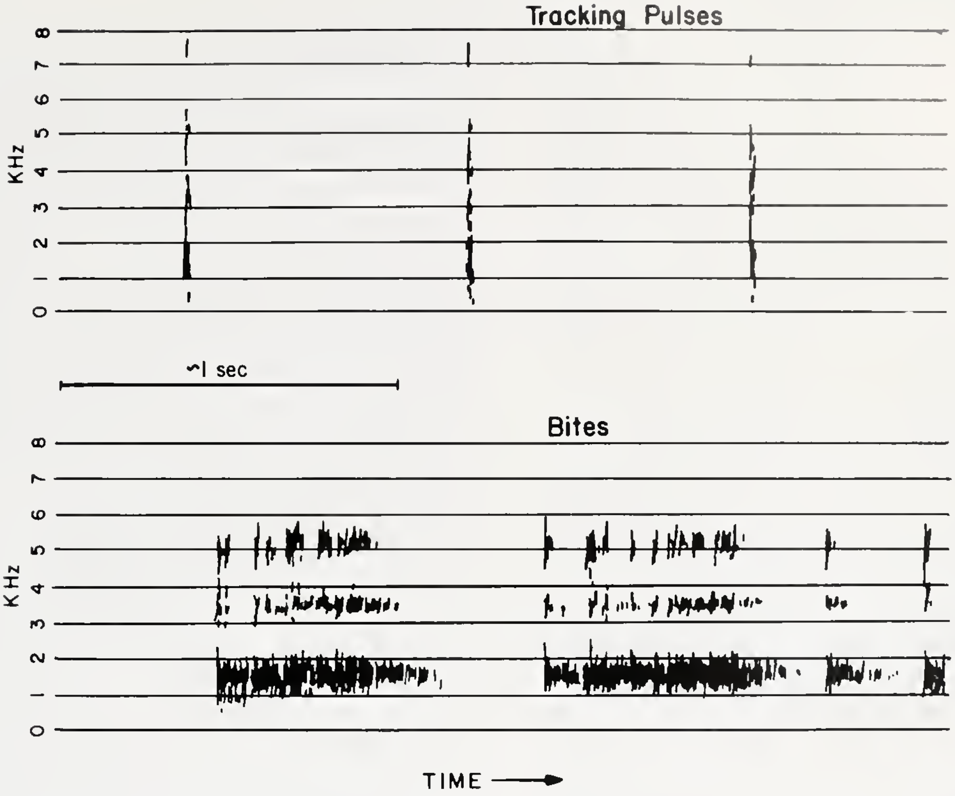

Figure 4. Sonographic representation of ultrasonic receiver output heard by operator. Top: tracking pulses (short "beeps"). Bottom: feeding signals (long "screeches"). Record shows two "bites."

weight loads similar to those caused by the barnacles sometimes found fouling crabs' carapaces. Despite the additional drag and weight, the only effect of a transmitter on a crab's behavior we could detect during careful comparative observations, of either the same crabs before and after tagging or between tagged and untagged crabs in laboratory aquaria, was an occasional attempt to preen or scrape the transmitter off. Tagged crabs otherwise exhibited normal behavior during all activities in the laboratory, including walking, swimming, digging, burying, and feeding. Moreover, when one large male released in the field ceased to feed, we retrieved him to discover that he was carrying a female in a precopulatory embrace, indicating that transmitters do not seriously interfere with sexual activity. With reasonable care in cleaning electrodes and electrode sites, no evidence of infection or serious irritation was evident. Transmitters would, of course, be cast off at ecdysis. One molt in the laboratory showed that the soft crab could withdraw from the electrodes with minimal damage, but had great difficulty in escaping from the old exoskeleton because the rubber patch prevented the dorsal carapace from lifting normally.

Laboratory observations of crabs equipped with transmitters verified that contraction of the mandibular muscle produced a signal distinct from the regular locating signal (Fig. 4). On the usual heterodyne-type (single conversion) ultrasonic receiver (*e.g.*, Sonotronics USR-5), the locating signal sounds like a short "beep," whereas the muscle contraction produces a more prolonged "screech." Only contractions of the mandibular muscle consistently produced a signal; even a major startle response produced, at most, a short "beep." No other behavior, neither escape responses, vigorous aggressive behavior, nor the frequent activity of the maxillipeds, produced spurious signals.

The theoretical threshold for biopotentials to trigger output pulses was calculated to be 60–80 μV. However, the programmable operational amplifiers become rather slow when operated at very low currents (10 μA), and the actual thresholds are somewhat higher. Minimum amplitude is about 250 μV for a 0.3 ms square-wave input pulse, falling to about 200 μV for a 4.5 ms input pulse. By increasing the time constant of the first amplifier stage the threshold can be lowered to about 100 μV, at the risk of passing increased low-frequency noise from electrode artifacts.

In the laboratory, instrumented blue crabs gave a single contraction of their mandibular muscle at irregular intervals averaging about an hour; rarely, a second con-

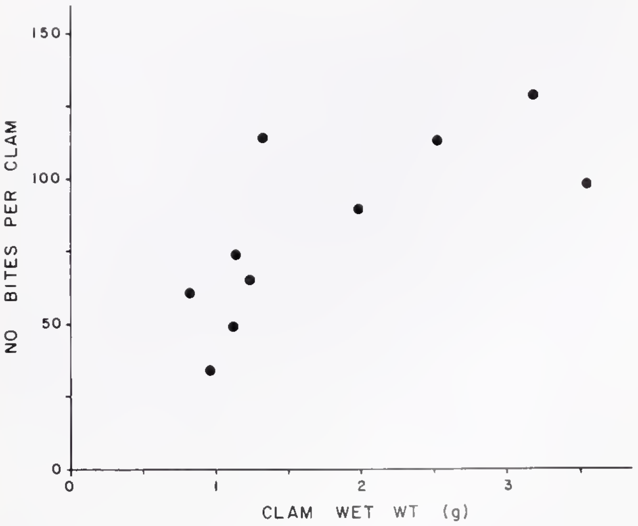

Figure 5. Number of bites required by a large male blue crab to consume prey items (clams, *Macoma balthica*) in the laboratory, as a function of prey size. (Bites = 22.7 × Weight + 42.0; corr. coeff. = .72). Feeding was conducted by dropping clams into a 100 l aquarium of Rhode River water at about 24°C, under subdued fluorescent light, and observing the crab through a blind while recording the signal from the transmitter.

traction followed. These single (and rare double) contractions were not associated with any obvious behavior and occurred while crabs were walking or inactive, while on the sediment surface or buried. Three or more closely spaced contractions of the mandibular muscle were always associated with feeding, and the signal from each contraction was distinct enough that the number of bites during feeding could be counted. As expected, feeding clams (*Macoma balthica*) to the prototype instrumented crab in a laboratory aquarium showed that the number of bites required to ingest a food item was positively correlated with prey size (Fig. 5).

As a field test of the method's feasibility, a large (153 mm carapace width, point-to-point) intermolt male blue crab equipped with one of the ultrasonic transmitters was released on 28 July 1986, in the Rhode River, a shallow (mean depth = 2 m, max. depth = 4 m), mesohaline subestuary of central Chesapeake Bay (see Hines *et al.*, 1987a, b, for descriptions of the site and population biology of blue crabs in the subestuary). The crab was trapped near the head of the subestuary and immediately brought to the nearby laboratory, where it was fitted with a transmitter and electrodes for the mandibular muscle, as described above. Once fitted, the crab was held and observed in a 200-l aquarium until it fed on a small piece of fish, confirming proper placement of the electrodes. Within 12 h of capture, the crab was released near its capture site. Using both directional and omni-directional hydrophones with an ultrasonic receiver in a small boat, the crab was tracked continuously for 96 h by a team of observers and the time of every bite was recorded.

The signals from the transmitter could be received from a range of 50–500 m, depending on weather conditions. Although a distinct thermocline did not form in the estuary, a diffuse temperature gradient developed in the water column during mid-day heat on calm summer days, causing refraction of the ultrasonic signals and greatly reducing range. The receiving range increased substantially when temperatures were cooler at night and/or when moderate breezes mixed the water column. The crab could be located readily to within one meter by triangulation with a directional hydrophone, and the boat was maintained within 10 m of the crab, anchoring when the crab did not move. The crab did not appear to respond to the presence of the boat.

The crab moved rapidly down the axis of the Rhode River and nearly out into Chesapeake Bay in the first 15 h after release, but stopped near the mouth of the river and spent the next 3 days meandering around an area approximately 200 × 300 m (Fig. 6). The point-to-point length of the track was 5130 m, for a net distance (shortest possible route) of 4213 m from release point to the point where tracking was terminated 96 h later. Average speed was 53 m/h with speeds ranging from 0 m/h during inactivity to 371 m/h during directional movement,

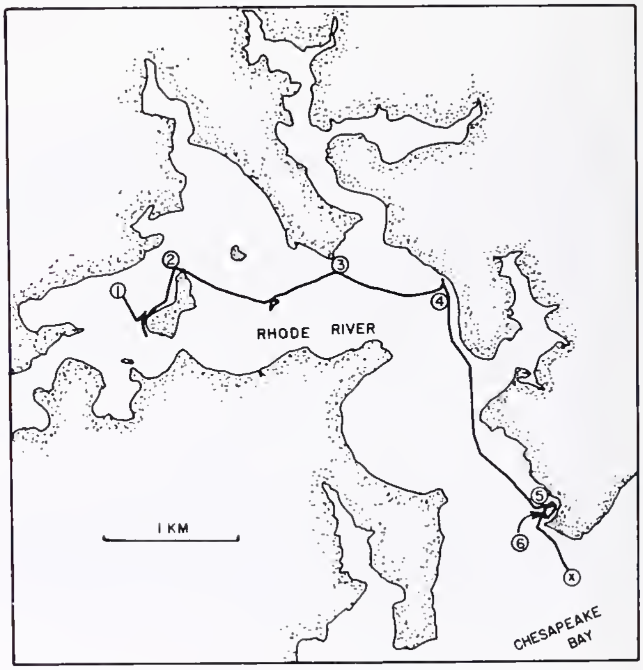

Figure 6. Track of the test blue crab in the Rhode River. Crab was released at position 1, moved rapidly to near the river mouth (positions 2–5), and meandered in a small area (positions 5–6) for the remainder of the tracking period. It was subsequently recaptured in a crab pot at "X."

when the crab was probably swimming. During the first 15 h when the crab was moving rapidly along the axis of the river, its average speed was 299 m/h; but during each of the next three days when it was meandering around near the mouth of the subestuary, its average speed was 8.6 m/h. Depths along the crab's track ranged from 0.5 m in sandy shallows near islands to 3 m on muddy bottoms of the main channel. No relationship between movement and time of day or tidal cycle was apparent.

The feeding record showed a highly variable pattern of 2–7 feeding bouts per day. The feeding bouts consisted of 15 to 2751 bites in 10 to 70 minutes and were readily apparent against a background of occasional single, apparently non-feeding muscle contractions as observed in the laboratory (Fig. 7). There were several periods lasting 10–170 minutes in which no bites were recorded. Feeding activity occurred at all times of the day and night, and there was no apparent relationship between feeding activity and the tidal cycle. Almost no feeding occurred when the crab was moving rapidly along the axis of the river, and most of the feeding bouts occurred when the crab meandered slowly around near the mouth of the subestuary.

The test crab was caught on 2 August 1986, in a commercial crab pot just outside the mouth of the subestuary, and was returned to us with the transmitter in working order.

Discussion

Although telemetry of an animal's location provides important data on movement rates, track, and habitat selection, biotelemetry of physiological function along with location can provide critical information on what animals are actually doing and how they are utilizing the habitat. Telemetry of muscular contractions provides data on the frequency, time, and location of the behavior(s) associated with a muscle's activity. The techniques we describe in this paper can be used on any muscle that is accessible for electrode implantation in an animal stout enough to carry the 5–6 g (immersed weight) telemetry package. Large arthropods are particularly well suited for this because many of their muscles originate on exposed exoskeleton, making insertion and immobilization of electrodes relatively easy. The specificity of the behavioral information derived from the telemetry will depend on the degree of specialization of the muscle's function. The techniques presented in this paper have allowed us for the first time to measure directly feeding activity of a free-ranging marine animal, by telemetering contractions of mandibular muscles.

Although the size of this transmitter and its simple electrodes will prevent its use on very small animals or very small muscles, it should be suitable for telemetering valuable behavioral information from a large number of decapod crustaceans (*e.g.*, tail flips or antennal movements in lobsters). We have successfully tested the transmitter in the laboratory on other muscles in the blue crab, including those of the fifth pereiopods (swimming

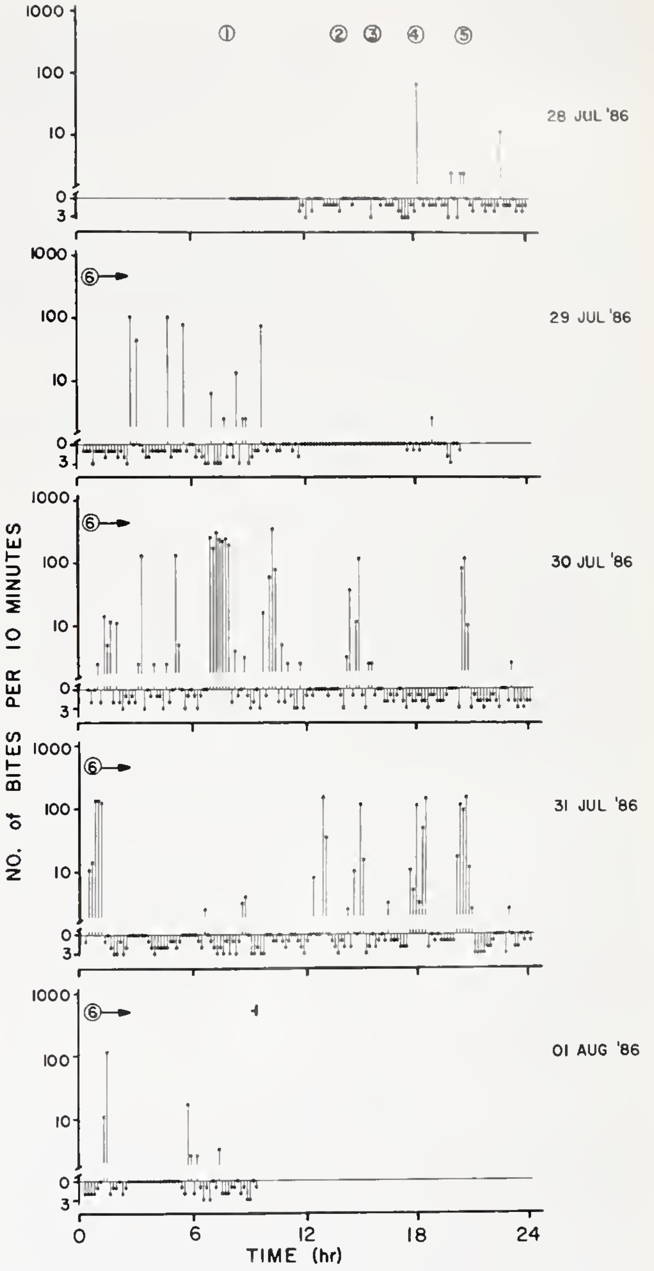

Figure 7. Four-day feeding record of the crab along the track shown in Figure 6. Circled numbers correspond to the numbered positions along the track. Lines extending upward from the axis show feeding; note that the number of bites is presented on a log scale. Isolated lines may be equated with feeding bouts. Grouped lines represent 1 or more bouts, lumped by the 10-min integrating period in this figure but resolvable in the original data. Lines below the axis represent the number of single (non-feeding) "bites" in each 10-min interval.

paddles) and the chelae. Signals from the former may be useful in telemetering walking, swimming, and burying; those from the latter, in distinguishing food manipulation and threat displays. Transmitters that will allow us to monitor several muscles simultaneously are currently under development.

How do blue crabs, as dominant predators, regulate the abundance and species composition of benthic invertebrates (Virnstein, 1976; Holland *et al.*, 1979; Laughlin, 1982; Hines *et al.*, in prep.)? In the Rhode River, about 50% of their diet is clams (*Macoma balthica* and *Mya arenaria*), and predation by crabs limits the abundance of clams during the warm summer months (Hines *et al.*, in prep.). Biotelemetry of feeding activity in our test blue crab revealed both the spatial and temporal patterns of foraging, as well as providing a rough correlation between the number of bites required to consume a food item and its biomass (*Macoma balthica* requiring about 50–150 bites).

Further analysis in the laboratory may allow us to differentiate types of food items by the biting pattern exhibited by crabs during feeding, because crabs pause in their chewing while they use their chelae to manipulate hard prey items like clams. The functional relationship between the number of bites and size of the prey item may provide information on the size-frequency distribution of prey and food consumption rate of free-ranging crabs in the field. Application of this "feeding telemetry" technique to numerous crabs will provide the statistical basis for assessing variation in foraging behavior among individuals, as well as measures of average feeding rates, diel rhythms, prey patch choice, and prey size (Hines *et al.*, in prep.; Nye, in prep.).

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The Intrinsic Origin of Bioluminescence in the Ascidian, *Clavelina miniata*[1]

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Abstract. Bioluminescence was observed in the ascidian, *Clavelina miniata* (Tunicata: Ascidiacea). This is the first report of luminescence in ascidians. A green light was evoked by mechanical stimulation, by increasing the concentration of K^+ ions, or by hypotonic conditions. The source of the light was a type of tunic cell (cell type II). This luminescence is attributed to an intrinsic system, not to bacterial symbionts.

Introduction

Bioluminescence in tunicates has been reported for pelagic species of the Thaliacea and Larvacea (Harvey, 1952; Herring, 1978), and there are two old reports of luminescence in ascidians: Landsborough (1842) in *Botryllus schlosseri* and Will (1844) in *Ciona (Phallusia) intestinalis.* The luminescence of these two ascidians is believed to have been caused by infections of luminous bacteria (Skowron, 1926; Harvey, 1952). However, recently we observed bioluminescence in *Clavelina miniata,* a colonial member of the Ascidiacea inhabiting sublittoral rocky shores (Watanabe and Tokioka, 1973). Our studies indicate that the bioluminescence of this ascidian is produced intrinsically, not by symbiotic bacteria.

Materials and Methods

Clavelina miniata colonies were reared on glass plates in rafts immersed in an inlet near the Shimoda Marine Research Center during 1983–1985. Fresh colonies were transferred from the rafts to aquaria in the laboratory for each experiment. Although the ambient seawater temperature range was 12°C–25°C, experiments were conducted at 18°C–20°C. Many individuals of this species grow in a dense aggregation (social type), but they never form a colony in the strict sense because newly formed individuals become physically separated by strobilization of the abdomen (Kamijo and Watanabe, unpub.) before the branchial aperture opens. Thus, individuals can be easily removed from a glass plate. Each individual is elongate, about 10 mm long in the extended state, and consists of a thorax and an abdomen enclosed in a gelatinous, transparent tunic (Fig. 1), which can be removed easily.

Preliminary observations showed that a remarkable green light was emitted from the whole tunic when placed in a hypertonic solution such as 2 *M* KCl or 2 *M* NaCl, or in a hypotonic solution. Also, mechanical stimulation of any point on the tunic resulted in localized light emission. Mechanical stimulation consisted of touching the tunic with a fine needle. The effects of these stimuli were then examined more thoroughly.

To determine the effect of K^+ ions or Na^+ ions while excluding the effect of osmotic pressure, mixed solutions of 0.54 *M* KCl and 0.54 *M* NaCl, isotonic with seawater, were prepared. The ratio of KCl to NaCl was varied from 0.0 to 1.0, at intervals of 0.1. For each ratio of solution, an individual was immersed in 1.0 ml of the mixed solution. Four individuals were examined. For each individual, light emission was recorded with an LKB Wallac luminometer, at 1-min intervals over a 10-min period, and the maximum value of relative light intensity was plotted.

Long duration light emission from the whole tunic was also observed when individuals were placed in a hypo-

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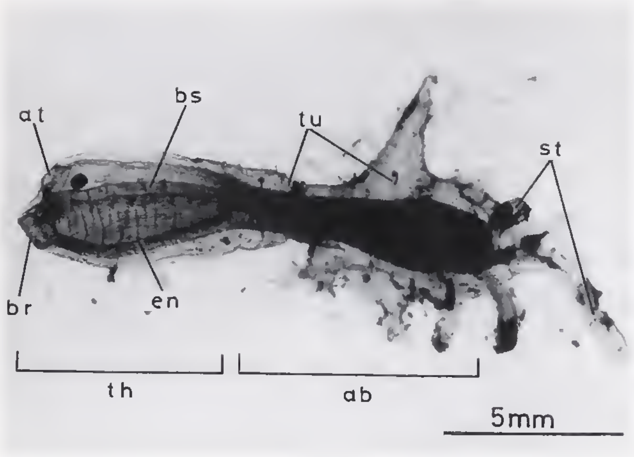

Figure 1. *Clavelina miniata*, growing on a glass plate, consists of a thorax and an abdomen enclosed in a transparent tunic. ab: abdomen; at: atrial siphon; br: branchial siphon; bs: branchial sac; en: endostyle; st: stolon; th: thorax; tu: tunic.

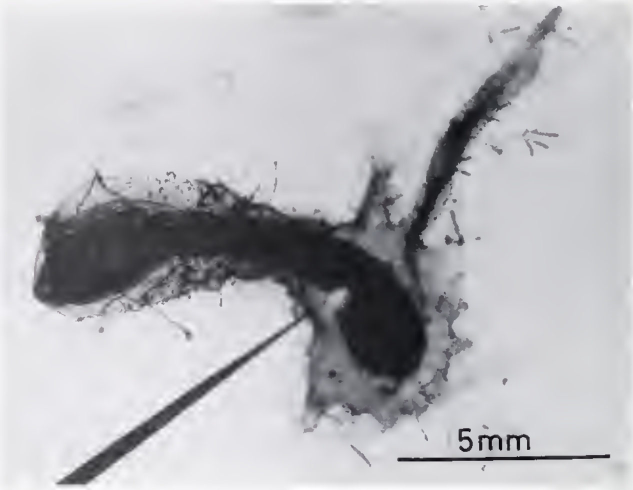

Figure 2. Luminescence of *Clavelina miniata* evoked by mechanical stimulation. The individual is touched by a fine needle. Note the strong luminescence emitted locally on tunic.

tonic solution. Three individuals were examined. Each was immersed in distilled water and records of relative intensity of light emission were made at 1-min intervals, until light emission was no longer detectable.

The thoracic tunic was chosen for microscopic observations, because of its high transparency. The tunic on a glass slide kept at room temperature begins to glow as the seawater evaporates, thus increasing the osmolarity. To establish the source of light emission, the position of flashes in the tunic was observed through an unilluminated microscope with a video camera and photomultiplier (Hamamatsu Photonics C-1000) and recorded on video film (SONY U-Matic VO-5800 Video Recorder). When light was no longer emitted, the tunic cells were illuminated and videotaped with the same apparatus. Cells in the tunic were examined using a Nikon microscope with Nomarski differential interference contrast optics.

Photographs were taken using Kodak Tri-X (ASA 400) or Kodak Plus-X (ASA 50) film.

Results

Stimulation evoking luminescence

(a) Mechanical stimulation. Mechanical stimulation with a fine needle could evoke luminescence from any part of the tunic of an intact individual. A strong green luminescence appeared locally at the site of stimulation (Fig. 2). The duration varied from 2 to 5 s.

(b) Change of ratio of K^+ ions and Na^+ ions. Relative light intensity of light emission was lowest for a K^+/Na^+ ratio of 0.1, and became higher as the proportion of K^+ ions was increased, especially >0.7 (Fig. 3).

(c) Hypotonic condition. In distilled water, the whole tunic of an individual began to emit a strong green light (Fig. 4). The intensity of light emitted varied for each individual, but reached a maximum after 2–5 min. The duration of light emission varied from 15 to 30 min (Fig. 5).

Source of light emission

Under a stereomicroscope (20×), the light emitted from the tunic following the stimuli mentioned above

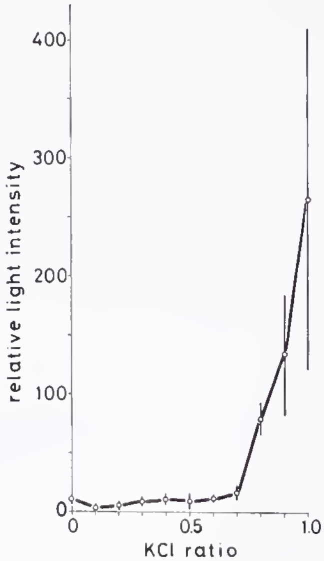

Figure 3. Mean maximum value of relative light intensity of luminescence emitted from the individuals during 10 minutes in solutions with different ratios of 0.54 *M* KCl and 0.54 *M* NaCl. The ratio of KCl to NaCl was varied from 0.0 to 1.0 at 0.1 intervals. Four individuals were examined for each ratio. Vertical bars indicate standard errors of the mean.

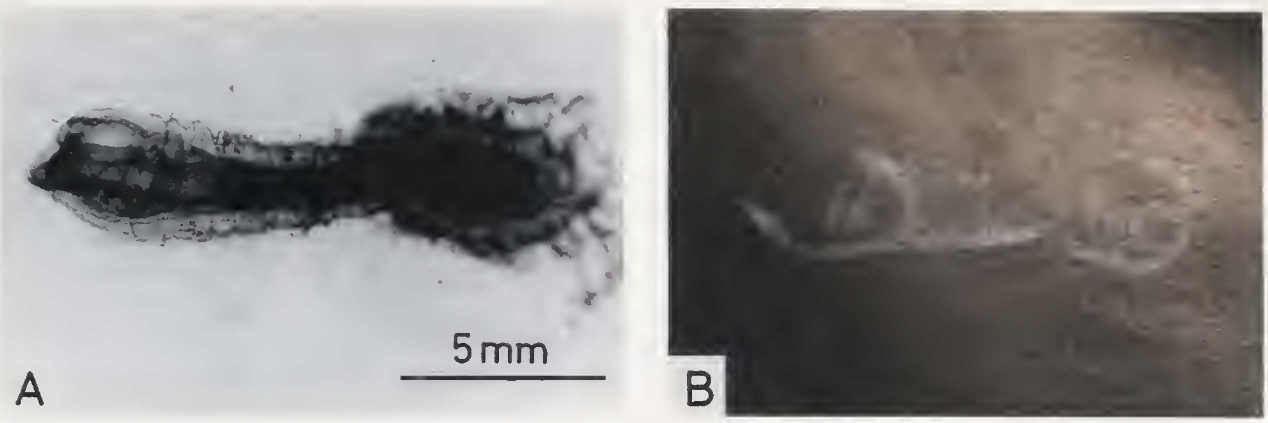

Figure 4. Luminescence of *Clavelina miniata* evoked by hypotonic conditions. (A) An intact individual in seawater; (B) an individual in distilled water, emitting light from the tunic continuously over a long duration.

was discerned to be groups of point-source lights in the tunic. The long duration light emission is due to the successive flashes of point-source lights over a period of time rather than the continuous luminescence of all the sources. On the other hand, the light emission by mechanical stimulation is a result of local luminescence of the point-sources at the stimulated site of the tunic. The point-source lights are apparently from the tunic cells. Cells are distributed densely in the tunic (Fig. 6A). Under examinations with Nomarski optics, these cells were classified by their morphological characteristics into three types:

I. Cells including 10–15 large amorphous vacuoles. Cell size: 20–25 μm long and 10–15 μm wide (Fig. 6B).

II. Flat cells including 1–10 spherical granules of 0.7–1.0 μm diameter. Cell size: 15–20 μm long and 7–10 μm wide (Fig. 6C).

III. Cells filled with many oval granules of 0.7–1.8 μm in the long axis. Cell size: 15–20 μm long and 10–15 μm wide (Fig. 6D).

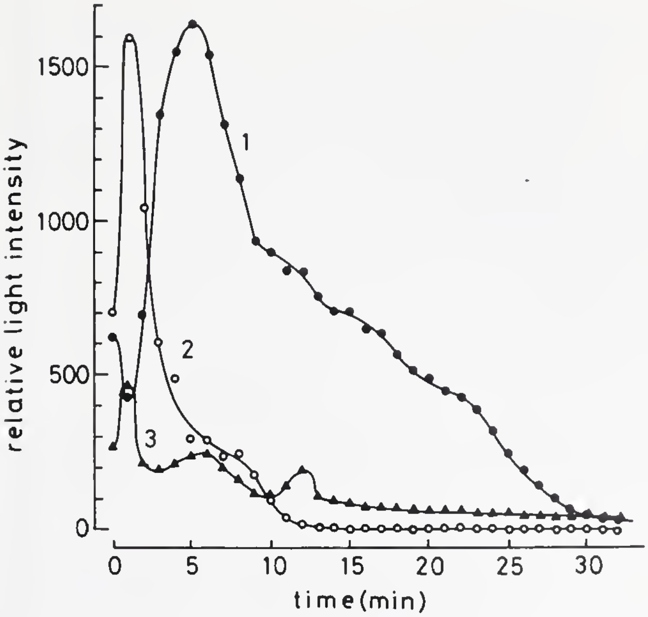

Figure 5. Changes in the relative light intensity of luminescence emitted by three individuals under hypotonic conditions. Numbers in the figure indicate different individuals.

Through an unilluminated light microscope, light emission appeared from a point-source, scattering strongly from the center before fading within 0.5–1.0 s (Fig. 7B–D). Comparing these figures with a figure of tunic cells (Fig. 7A), it is clear that the light was emitted from a definite type of cell: cell type II. In numerous preparations, we observed no light emission from other cell types.

Discussion

Although this is the first report of intrinsic bioluminescence in ascidians, there are several papers describing the luminescence of pelagic species in the Thaliacea and Larvacea.

Generally, three types of animal luminescent responses can be distinguished. These are extracellular luminescence, intracellular luminescence, and luminescence due to symbiotic bacteria (Nicol, 1960). In the case of luminescence due to bacterial symbionts, the presence of luminous bacteria can be established by microscopic observation, culturing, continuous light emission, and the occurrence of bacterial luciferase (Nicol, 1960; Leisman *et al.*, 1980; Nealson and Hastings, 1980). However, in *Pyrosoma* (Tunicata: Thaliacea), the distinction between symbiotic and intrinsic light emission was not easily made. It has been suggested that the source of intermittent luminescence is not symbiotic bacteria (Harvey,

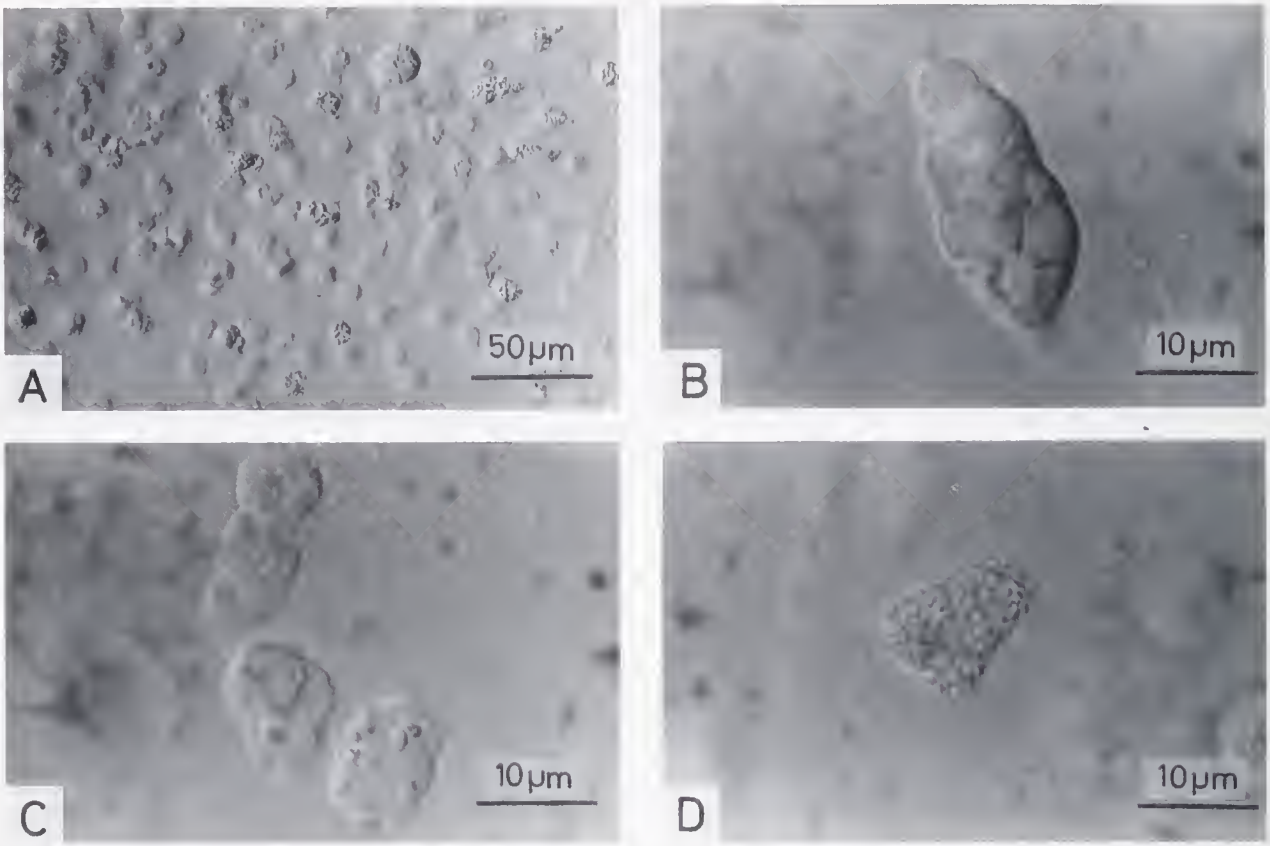

Figure 6. Portion of the thoracic tunic of *Clavelina miniata* viewed with Nomarski optics showing the distribution of cells in the tunic (A); a tunic cell of type I (B); tunic cells of type II (C); and a tunic cell of type III (D).

1952). According to Nicol (1960), the luminescence of *Pyrosoma* apparently differed from that of luminous bacteria because it was intermittent, excitable by tactile, electrical, and photic stimuli, and quenchable by illumination. However, bacteria-like bodies packed in the luminous organ have been observed (Mackie and Bone, 1978), and bacterial luciferase has been found in it (Leisman *et al.*, 1980; Nealson and Hastings, 1980). The luminescence of *Pyrosoma* may be controlled by the packaging of bacterial symbionts within the host cells (Mackie and Bone, 1978; Anctil, 1979; Nealson and Hastings, 1979, 1980).

On the other hand, in the larvaceans, oikopleurids possessing oral glands produce endogenous light upon mechanical stimulation (Fredriksson and Olsson, 1981; Galt *et al.*, 1985). In *Oikopleura dioica* and *O. labradoriensis*, light was produced from 1–2 μm clusters of fluorescent granules (Sykes, 1980; Galt and Sykes, 1983). However, the light emitting mechanism in larvaceans is unknown (Galt and Sykes, 1983) and bacterial luciferase has not been found (Galt, 1978; Hastings, 1983). The relationship between the luminescent mechanisms of *Clavelina* and larvaceans is unknown, although they share the characteristics that light can be evoked from a point-source by mechanical stimulation.

The mechanism of luminescence in *Clavelina* is probably caused by an intrinsic mechanism. The apparently continuous luminescence was found to be the result of successive flashes of point sources of light. Furthermore, the emission of light could be caused by various modes of stimulation such as mechanical stimulation, increase of extracellular K^+ ions, and hypotonic conditions.

The results obtained in the observation through the unilluminated microscope show that one type of tunic cell of *Clavelina*, the type II cell, luminesces when stimulated appropriately. This type of cell can be easily discriminated from the other two by its morphological characteristics. We do not yet know the chemical nature of the luminescence.

In the ascidian *Clavelina*, light emission was strong when the K^+ ratio was high, so the luminescence may be caused by the asymmetrical distribution of anions and cations across the cell membrane. This type of luminescence has been reported for the coelenterates, *e.g.*, *Vere-*

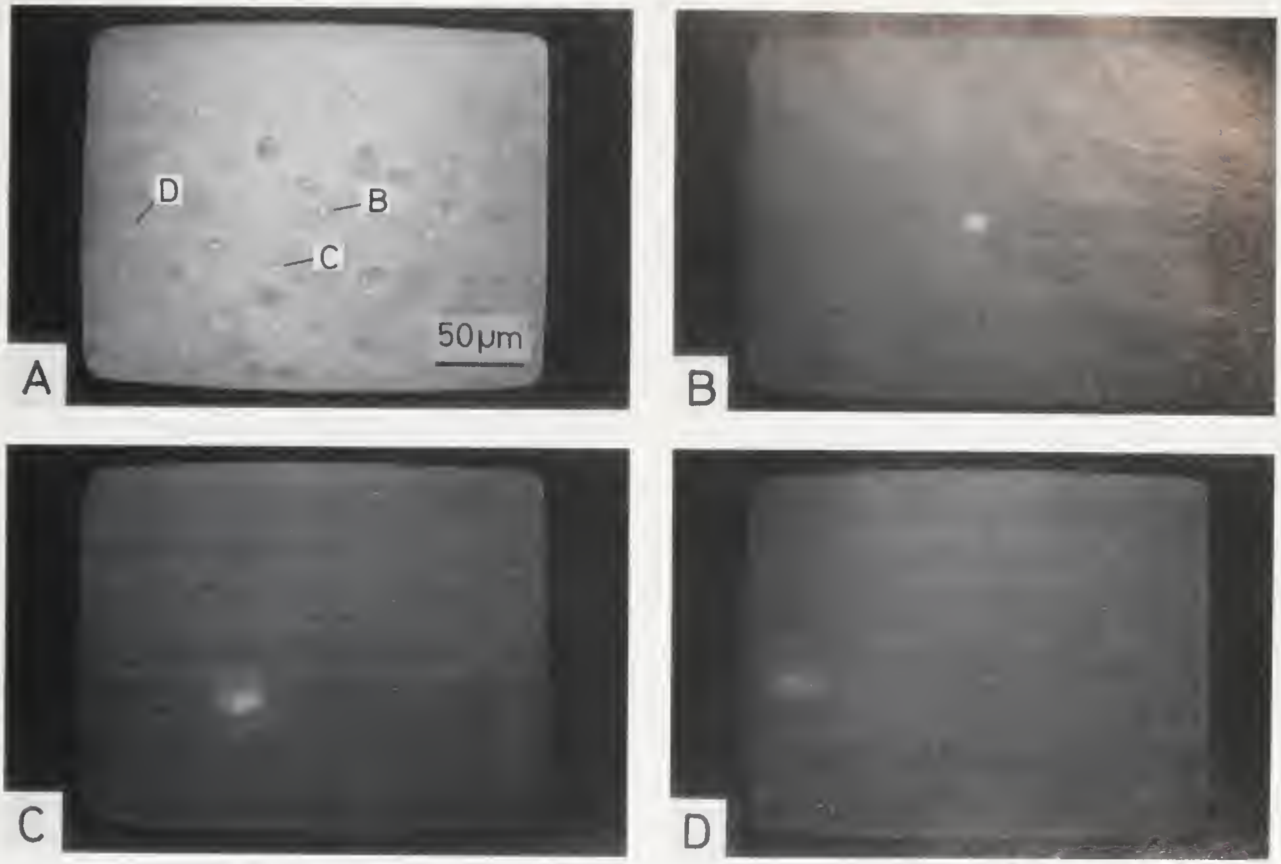

Figure 7. The source of light emission in the tunic. Tunic cells recorded on video tape through an illuminated microscope showing cell types I, II, and III (A). Individual type II cells (B, C, D as indicated in A) emitting light recorded on video tape through a fixed unilluminated microscope with a video camera and a photomultiplier (B–D).

tillum and *Renilla* (Henry and Michelson, 1978; Henry and Ninio, 1978). In this case, an asymmetrical distribution of cations and anions on the membrane of the lumisomes was attributed to an influx of Ca^{2+} ions, which triggers luminescence. Ca^{2+} ions can pass through the cell membrane if there is a higher proportion of Na^+ ions inside the cell, and a higher proportion of K^+ ions outside. Mechanical stimulation may also change the ion permeability of the membrane, inducing luminescence.

The luminescence observed in hypotonic conditions may be caused by the rupture of cell membranes after osmotic uptake of water, thereby releasing the luminous substance. A similar phenomenon is known in the lumisome system: lumisomes in distilled water can emit light (Anderson and Cormier, 1973).

Further experiments are required to clarify the chemical nature of the luminous substance and the mechanisms of the luminescence, as well as the fine structure of the type II cell.

Acknowledgments

We thank Professor I. Yasumasu and Dr. H. Namiki for their advice and encouragement throughout this study. We also thank Professors H. Sakurai and S. Ishiwata for their helpful suggestions and for allowing us the use of facilities at Waseda University, and the staff of the Shimoda Marine Research Center for their assistance throughout this study.

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Reference: *Biol. Bull.* **176:** 63–71. (February, 1989)

Abstracts of Papers Presented at the Marine Biological Laboratory: Northeastern Regional Conference on Developmental Biology 17–20 November 1988

The conference was organized by Marian R. Goldsmith, University of Rhode Island, and Nathan H. Hart, Rutgers University. Financial support was provided by Allied Fisher Scientific, the American Society for Zoologists, The Council for Tobacco Research, the National Science Foundation, Pharmacia, Inc., and the Society for Developmental Biology. The Best Student Presentation Awards were sponsored by the American Society of Zoologists.

The Cytoskeleton and Early Development

Expression of villin during differentiation of mouse visceral endoderm and F9 teratocarcinoma cells. R. M. EZZELL, M. M. CHAFEL, AND P. T. MATSUDAIRA (Whitehead Institute and Dept. of Biology, MIT).

Villin is a member of a family of actin-binding proteins that regulate the length and assembly of actin in a Ca^{2+}-dependent manner. In the adult, villin is a major structural component of microvilli in brush border epithelium. In examining the pattern of villin expression in the mouse (using immunofluorescence/confocal scanning and immunoelectron microscopy), we find that villin first appears at day 5 of development in the primitive endoderm, a cell layer lining the blastocoelic surface of the inner cell mass. The primitive endoderm cells that express villin differentiate into the visceral endoderm, an extraembryonic tissue involved in absorption and secretion. Villin is concentrated in the apical surface in the visceral endoderm in a pattern similar to its distribution in the intestine. Villin is also expressed in F9 teratocarcinoma cells when cultured as aggregates in the presence of retinoic acid. Villin appears in the outer cell layer of the F9 aggregates, which differentiates into an epithelium resembling the visceral endoderm. Fimbrin, an actin-bundling protein also found in brush border epithelium, is expressed throughout development (including the primary oocyte) and in undifferentiated F9 cells. Fimbrin co-localizes with villin in the apical surface in the visceral endoderm and in F9 cells treated with retinoic acid. The expression of villin and fimbrin in tissues that have similar absorptive functions supports a role for these two actin-binding proteins in the assembly and regulation of the microvillus cytoskeleton, and shows that villin can be used as a cytoskeletal marker to examine the differentiation of the visceral endoderm in the embryo and in F9 teratocarcinoma cells.

Dynamics of spectrin in sea urchin oogenesis and embryogenesis. D. J. FISHKIND[1], E. M. BONDER*, S. M. BEAULIEU, J. H. HENSON, AND D. A. BEGG (Harvard Medical School, Boston, MA, *Rutgers University, Newark, NJ).

The "membrane-cytoskeleton" of the sea urchin egg and embryo has long been recognized as a dynamic system involved in such processes as fertilization, cytokinesis, and differentiation. To understand more clearly "how" it functions in development, we have mapped the developmental expression and cellular redistribution of egg spectrin (SP) during oogenesis and early embryogenesis. During oogenesis, SP is found at the plasma membrane (PM) of oogonia, and is later expressed in the ooplasm of early oocytes where it assembles onto newly synthesized organelles. In mature eggs, SP is enriched in the cortex, appearing to encoat both cortical granules and cytoplasmic vesicles. Upon fertilization, SP redistributes to the PM, and SP-coated vesicles become firmly associated with the cortex. These Sp-vesicles concentrate to the cleavage furrow, later repolarize to the cortex of the blastomeres, and at times appear to fuse with the PM at points of cell-cell contact. In later stage embryos (morula to plutei), SP is predominantly found along the apical and basolateral PM of embryonic epithelial cells. These results suggest that SP's dynamics in development is indicative of its role in membrane biogenesis, vesicle trafficking, and cellular differentiation.

Dynamics of alpha-actinin and myosin in developing muscle cells. Y.-L. WANG (Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, MA).

Fluorescently labeled alpha-actinin and myosin were microinjected into cultured chick embryonic myotubes to examine whether these proteins can associate with developing sarcomeres and, if so, to follow the distribution of these proteins during myogenesis. Both proteins dispersed from the site of microinjection and became incorporated into sarcomeres of mature myotubes within 30 min, even though myosin has a relatively low solubility under physiological salt conditions. Incorporation was also observed with light meromyosin but not heavy meromyosin, suggesting that the C-terminal region was responsible for the process whereas actin-myosin interactions were not involved. Alpha-actinin became associated with diffuse aggregates in immature cells. The aggregates coalesced into better defined Z-line precursors, which then condensed into well-defined Z-lines. Nascent Z-lines of neighboring myofibrils underwent continuous association and dissociation, allowing long-range alignment to be achieved in a trial and error fashion.

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[1] Winner, Best Student Presentation Award (platform).

Microtubule dynamics during anaphase. P. WADSWORTH, E. SHELDEN, J. RUPP, AND C. L. RIEDER. (Dept. of Zoology, Univ. of Mass, Amherst, MA, and N. Y. State Dept. of Health, Albany, NY).

Biotinylated tubulin has been microinjected into mitotic PtK1 cells to examine microtubule (MT) dynamic behavior throughout mitosis. The location of the biotinylated tubulin subunits was detected using antibiotin antibodies and light or electron microscopic localization techniques. Video analysis of injected cells reveals that chromosome motion continues at rates comparable to control, uninjected cells. In early anaphase cells, dim fluorescence is observed throughout the spindle and asters. In the electron microscope, gold labeled MTs interspersed with unlabeled MTs are observed; some labeled MTs interact with the kinetochore. These observations demonstrate that MT turnover continues during anaphase, and suggest that kinetochores may capture and release MTs during anaphase chromosome motion. In cells injected later in anaphase, segments of new MT elongation are observed throughout the interzone and in the aster. In cells in which a midbody is forming, label is observed first in the asters, not in the midbody region, at early times post-injection. These techniques provide new information concerning the sites and relative rates of tubulin incorporation in anaphase cells.

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Regulation of anaphase chromosome motion in Tradescantia *stamen hair cells by Ca and Ca-related signaling agents.* D. H. ZHANG, D. A. CALLAHAM, AND P. K. HEPLER (Department of Botany, Univ. of Mass., Amherst, MA).

The increase in free [Ca] observed as chromosomes move to the poles in *Tradescantia* stamen hair cells (Hepler & Callaham 1987, *J. Cell Biol.* **105:** 2137) supports the idea that the ion regulates the rate of motion. We have now iontophoretically injected Ca or agents that affect the intracellular free [Ca] and measured their effect on the rate of chromosome motion. A Ca injection of 1 nA for 10 s (approximately 1 μM) causes a transient (20 s) increase in the rate of motion from 1.01 μm/min to 2.10 μm/min. Injection of a high level of Ca (4 nA, 10 s), in contrast, causes chromosomes to stop and even regress slightly before resuming normal motion. Injection of Ca-saturated dibromo-BAPTA produces an acceleration of motion similar to a Ca injection. Both Ca-free BAPTA and EGTA inhibit chromosome motion. The injection of the Ca-related signaling agent GTPγS produces the same effect as a low level of Ca, whereas GTP or IP$_3$ yield only a brief increase in motion, possibly due to their rapid metabolic inactivation. The results show that movement of the chromosomes to the poles in anaphase is markedly sensitive to the level of Ca in the physiological range.

Supported by NSF grants DCB 85-02723 and DCB 88-01750.

Localization and developmental dynamics of a calsequestrin-containing ER in sea urchin eggs. J. HENSON, S. BEAULIEU, D. FISHKIND, E. BONDER*, D. LEBECHE⁺, B. KAMINER, M. TERASAKI#, AND D. BEGG (Harvard Med. Sch. Boston, MA, *Rutgers U. Newark, NJ, ⁺Boston U. Sch. of Med. Boston, MA, and #The Marine Biol. Lab. Woods Hole, MA).

The storage of intracellular calcium (Cai) in the SR of muscle is facilitated by the Ca-binding protein calsequestrin (CS). The means of sequestering Cai in the ER of nonmuscle cells remains unknown. Using an antibody to the sea urchin analog of CS, we performed immunocytochemical localizations on eggs and early embryos. In semithin frozen sections of unfertilized eggs, the CS antibody stains a tubuloreticular network throughout the cytoplasm. CS staining of isolated egg cortices confirms the presence of a polygonal, tubular network similar to ER networks in other cells and to the cortical network stained with the membrane dye DiIC16. In mitotic eggs, the CS staining concentrates in the asters of the mitotic apparatus where ER is known to congregate. In addition, immunoelectron localization shows CS to be present within ER tubules. Finally, in centrifugaly stratified eggs, CS staining concentrates in the clear zone: a layer rich in ER and thought to be the site of Cai release upon fertilization. These results indicate that the CS-ER may represent the Cai sequestration system involved in the regulation of fertilization and cell division of sea urchin eggs.

Cortical inheritance in ciliates, membrane microdomains, and prions: models for extranucleic acid-based inheritance. G. W. GRIMES* AND K. H. AUFDERHEIDE# (*Department of Biology, Hofstra University, Hempstead, N.Y. 11550, and #Department of Biology, Texas A&M University, College Station, TX 77843-3258).

The ciliate cortex has been the subject of numerous investigations regarding information which is present to organize the new pattern during development. Especially significant are the data showing inheritance patterns that are independent of changes typically considered to be "mutagenic." The "inverted kinety," as well as standard doublets and mirror-imaged "Siamese Twins" are but a few of the examples available to document this statement.

We suggest that phenomena which have become of popular interest represent common developmental mechanisms of major significance. Membrane "microdomains" are widely accepted. Prions (which probably represent inherited allosteric changes of a nuclearly determined gene product) are analogous, if not homologous, to mechanisms involved in the determination of inheritance patterns of the ciliate cortex. We have defined these phenomena as directed assembly and patterning.

Supported by grants from the NSF and HCLAS to GWG and from the NIH to KHA.

Patterning Events Controlled by Homeobox Genes

The regulation of zerknullt (zen), a homeobox gene required for the dorsal-ventral pattern of Drosophila. HELEN DOYLE (Department of Biological Sciences, Columbia University, New York, NY).

Zerknullt (*zen*), a homeobox gene located in the Antennapedia complex (ANT-C), is required for the establishment of the dorsal-ventral pattern during *Drosophila* embryogenesis. The phenotype seen in *zen* mutant embryos can be explained as an alteration of cell fate such that the dorsal-most cells of the blastoderm follow a more ventral pathway of development. Thus, *zen* appears to be a selector gene for a subset of cells along the dorsal-ventral axis. *Zen* shows a simple dorsal on-ventral off pattern of expression in early embryos, and this pattern is refined such that *zen* is expressed only in the dorsal-most cells by gastrulation. We would like to understand how *zen* expression is regulated by other genes involved in the establishment of dorsal-ventral polarity. We have analyzed *zen* expression in many maternal and zygotic dorsal-ventral mutants to identify possible candidates for trans-acting regulators of *zen*. By P-element transformation studies, we have also identified regulatory sequences upstream of *zen* that are required for its normal expression pattern, and these regulatory elements may interact directly with some of the other dorsal-ventral gene products.

A novel Drosophila *homeobox gene specific to gut muscle cells.* M. BARAD*, T. JACK†, R. CHADWICK†, A. ERLEBACHER†, AND W. MCGINNIS† (Depts. of *Human Genetics and †Molecular Biophysics and Biochemistry, Yale University, New Haven, CT).

The homeobox is an approximately 180 bp region of homology found in many genes that are spatially and temporally restricted in their expression during the development of both vertebrate and invertebrate animals. The homeobox gene family of *Drosophila* appears to control a variety of position-specific patterning decisions during embryonic and imaginal development. Most of these patterning decisions determine groups of cells on the anterior-posterior axis of the *Drosophila* germ band. We have isolated a novel homeobox gene from *Drosophila*, designated H2.0. H2.0 has the most diverged homeobox so far characterized in metazoa, and, in contrast to all previously isolated homeobox genes, H2.0 exhibits a tissue-specific pattern of expression. The cells that accumulate transcripts for this novel gene correspond to the visceral musculature and its anlagen. Homozygous deletions including H2.0, as well as point mutations tentatively assigned to the locus, result in embryos and larvae which fail to form a normal gut. Mutant guts show a variety of effects ranging from restricted areas of varicosity in the sinuous tubes of gut, to replacement of all gut tubes by a single large sack filling the entire coelom of the animal.

Cis- and trans-regulation of a chorion gene. M. J. SHEA, B. D. MARIANI, AND F. C. KAFATOS (Dept. of Cellular and Developmental Biology, Harvard University).

Choriogenesis in *Drosophila melanogaster* involves the tightly controlled regulation of a number of major chorion genes during oogenesis. Each gene in this small multi-gene family exhibits a characteristic pattern of expression marked by sex, tissue, temporal, and quantitative specificity. We have initially chosen to study the late expressing s15-1 chorion gene. Analysis of internal deletion and linker scanner constructs spanning the promoter have delineated important cis-acting sequences. In addition, using a follicular nuclear extract, we observe a number of binding species which recognize these cis-acting sequences. Thus far, we have identified an early stage repressor, a late stage activator, and an essential activator binding to the chorion-specific TCACGT hexamer. Characterization and cloning of these factors are now underway. This, along with further *in vivo* experiments, will allow us to gain insight both on how a chorion gene is regulated and how the overall process of choriogenesis occurs.

Spatial regulation of homeobox fusions in the embryonic central nervous system of transgenic mice. J. ZAKANY, C. K. TUGGLE, M. PATEL, AND M. C. NGUYEN-HUU (Depts. Urology and Microbiology, Columbia Univ., NY).

The spatially restricted expression of mammalian homeobox genes in the embryonic central nervous system (CNS) provides an opportunity to study the basis of spatial gene regulation in mammalian development. Here we define a regulatory region of the murine *Hox 1.3* gene that mediates such a region-specific expression pattern. The *Hox 1.3* gene contains two exons, encodes a putative protein of 270 amino acids, and is expressed preferentially in the spinal cord at midgestation. We have analyzed transgenic mice containing various *Hox 1.3* DNA fragments fused to reporter sequences, such as a human growth hormone gene fragment or the *E. coli* lacZ structural gene. As shown by RNase protection assays or by *in situ* analyses of beta-galactosidase activity, several hybrid genes are expressed in the embryonic CNS in a spatially restricted manner, along both the rostrocaudal and dorsoventral axes. A 912 nucleotide sequence located immediately upstream of the *Hox 1.3* coding sequence is sufficient to direct expression to the dorsolateral cells of the brachial spinal cord.

Environmental and Physiological Effects on Plant Development

Control of cereal embryogenesis and the regulation of gene expression by abscisic acid (ABA), RALPH S. QUATRANO, WILLIAM R. MARCOTTE, JAMES C. LITTS, CHRISTOPHER C. BAYLEY, AND SONJA A. SCHMITZ (Central Research & Development Department, Experimental Station, E402, E. I. du Pont de Nemours & Co., Wilmington, DE 19898).

Over a 3–5 day culture period in the presence of ABA (1–100 μM), immature wheat embryos increase in fresh and dry weight and undergo an accelerated but normal morphogenesis and accumulation of mature embryo proteins. In the absence of ABA, this maturation program is not observed and the embryo germinates into a normal seedling. Some proteins that accumulate in the presence of ABA have been indentified and include the lectin wheat germ agglutinin, an abundant protein found in the mature embryo (Em protein) and the globulin storage proteins. Characterization of these genes and the pattern of accumulation of their respective proteins and mRNA's during grain development, and in culture with ABA, revealed that regulation of these genes by ABA occurs at the level of transcription and mRNA stability. Constructs of the intact and deleted 5′ as well as the 3′ regions with the glucuronidase (GUS) reporter gene have been tested in transient assays using protoplasts from monocots and dicots. The same constructs, as well as others containing the entire gene, were used in *Agrobacterium*-mediated gene transfer to selected dicots. Results from both the transient and stable transformation assays will be discussed in relation to the cis-acting sequences involved in the ABA regulation.

A novel method of tagging developmental genes in Arabidopsis *utilizing the T-DNA of* Agrobacterium. KENNETH A. FELDMAN (Central Research, E. I. DuPont, Wilmington, DE).

A population of 1400 transformants have been generated by infecting seeds of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*. The *Agrobacterium* strain contains a cointegrative Ti plasmid with a dominant selectable marker, kanamycin resistance, located between the T-DNA borders. The number of functional T-DNA inserts has been ascertained in 162 of these lines. Thirty to forty seeds from lines segregating for the Kan^R marker have been screened for altered phenotypes that segregate in a Mendelian fashion. A variety of altered phenotypes have been observed. These include seedling-, embryo-, and gametophyte-lethals, dwarf, glabrous, and flower mutants, among others. The latter three mutations have been shown to be linked to the T-DNA insert, consistent with the hypothesis that the insert has disrupted a gene which in the homozygous state confers an altered phenotype to the plant. The dwarf mutant will be discussed.

Patterns of leaf development in C4 plants. J. A. LANGDALE, E. MILLER, AND T. NELSON (Biology Department, Yale University, New Haven, CT).

The leaves of C4 plants exhibit fine-scale positional control of gene expression. Adjacent photosynthetic bundle sheath and mesophyll cells differ dramatically in their expression patterns of the C4 pathway genes. We present molecular and physiological evidence that supports a model in which both light and a positional effector locally surrounding each vein act to confine expression of each C4 gene to either BS or M cells. *In situ* hybridization analyses of C4 gene expression in early leaf primordia show that this positional control begins at the time veins are formed.

Photorespiration and light act in concert to regulate the expression of the nuclear gene for chloroplast glutamine synthetase. J. W. EDWARDS AND G. M. CORUZZI (Laboratory of Plant Molecular Biology, Rockefeller University, New York, NY 10021).

Several isoforms of glutamine synthetase (GS) are present in pea that differ in their subcellular location and pattern of expression. The GS isoforms are encoded by different nuclear genes that comprise a small gene family. One GS isoform (GS2) is located within the chloroplast where it functions in part to assimilate the ammonia generated during photorespiration. We have used gene-specific probes to study the external and metabolic factors involved in GS2 expression. The GS2 mRNA is present at low levels in etiolated pea and accumulates to high levels upon exposure to light. The light-induced accumulation of GS2 mRNA is mediated in part by the chromophore phytochrome. However, maximal accumulation of GS2 mRNA in white light involves metabolic induction by photorespiratory ammonia. Photorespiring peas (grown in air) contain four-fold more GS2 mRNA than nonphotorespiring plants (grown in 2% CO_2). The GS2 promoter has a complex structure that may reflect intricate gene regulation. Fragments of the GS2 promoter are being tested in transgenic plants to define the cis-acting DNA elements required for tissue-specific and light-regulated expression.

Expression of nodule-specific genes in ineffective alfalfa nodules induced by mutant strains of Rhizobium meliloti. JOANNA H. NORRIS[1], ANN M. HIRSCH[2], LISA A. MACOL, LINDA J. UTRUP[1], AND ANDREW CARY[1] ([1]Department of Botany, University of Rhode Island, Kingston, RI, and [2]Department of Biology, UCLA, Los Angeles, CA).

Analysis of ineffective symbiotic infection of alfalfa by mutant strains of *Rhizobium meliloti* has proven useful in elucidating the early stages of root nodule development. Only early nodule-specific gene products (nodulins) are detected in ineffective nodules induced by various exopolysaccharide (*exo*) mutants of *R. meliloti* or *Agrobacterium* transconjugates carrying the symbiotic megaplasmid of *R. meliloti*. Additional nodulins, including leghemoglobin, are expressed in ineffective nodules induced by the spontaneous mutant *fix*21.

We are also investigating nodule-specific gene expression in *Melilotis alba*, white sweetclover, a related diploid species also infected by *R. meliloti*. Ten sweetclover mutants isolated by Dr. Tom LaRue of the Boyce Thompson Institute (Cornell University, induced the *nod* genes of *R. meliloti*, although these mutants fail to form nodules.

Regulation of Development by Hormones and Growth Factors

Evolution and coordinate regulation of the chicken vitellogenin genes. J. B. E. BURCH, R. SILVA, AND A. FISCHER (Fox Chase Cancer Center, Philadelphia, PA).

Three vitellogenin proteins are expressed in the livers of chickens in response to elevated levels of estradiol. The major vitellogenin gene (VTGII) was previously cloned and sequenced. We now report the isolation of genomic clones that span one of the two known minor vitellogenin genes (VTGIII). A Southern cross comparison of 45 Kb from this locus with 45 Kb from the VTGII locus revealed that a pseudogene for VTGII (ψVTGII) lies 1.4 Kb upstream of the VTGIII gene. The converse is also true; namely, a previously unsuspected pseudogene for VTGIII (ψVTGIII) lies 1.4 Kb downstream of the VTGII gene. DNA sequence comparisons indicate that the initial gene duplication event that gave rise to tandem vitellogenin genes occurred early in avian evolution since the first three exons for the VTGIII and VTGII genes are 37% divergent. Similarly, only five small islands of homology are apparent in the respective upstream regions. Two of these regions are of known functional importance. We also show that the VTGIII promoter has deletions over two sequences that were previously shown to be conserved between the VTGII gene and four *Xenopus laevis* vitellogenin genes. The fact that one of these is an enhancer probably contributes to the VTGIII gene being expressed an order of magnitude less efficiently than the VTGII gene.

Tissue-specific hypersensitive sites within two coordinately regulated loci map to a conserved 91bp sequence located more than 5 Kb away from each promoter. J. B. E. BURCH, R. SILVA, AND A. FISCHER (Fox Chase Cancer Center, Philadelphia, PA).

We previously showed that the major vitellogenin gene (VTGII) is marked by tissue-specific nuclease hypersensitive sites before the gene is activated by estradiol. One of the most prominent sites maps to a sequence 8.7 Kb downstream of the VTGII promoter. We now report that a second copy of this sequence is present 5.6 Kb upstream of a minor vitellogenin gene (VTGIII). Within the VTGIII locus this 91 bp sequence exists as a remnant fragment of a VTGII pseudogene. The sequence is also marked by a tissue-specific hypersensitive site in this context. *In vitro* DNA binding studies indicate that a factor present in nuclear extracts can interact specifically with this sequence. In addition to this site, we identified four other sites within the VTGIII locus in hormone-naive birds, as well as two sites that are induced in response to estradiol. One of the induced sites maps to the promoter whereas the other maps 7.5 Kb upstream of the VTGIII gene. These data suggest that sequences located far away from the VTGIII promoter may be relevant to the estrogen-dependent liver-specific expression of this gene and that one such distal sequence may be involved with the coordinate expression of the VTGIII and VTGII genes.

Differentiation of proopiomelanocortin (POMC) cells during pituitary development. J. E. PINTAR, D. I. LUGO, AND R. SCOTT (Dept. Anat. & Cell Bio., Columbia P&S, NY).

Anterior (AL) and intermediate (IL) lobe POMC cells originate from different regions of Rathke's pouch. In the adult pituitary gland, these two POMC cell populations exhibit differences in the extent of post-translational processing and regulation of transcription and secretion. We have begun to investigate when these lobe-specific differences are acquired during development. Analysis of POMC-derived peptides has shown that IL-like proteolytic cleavages of the POMC precursor occur during stages when POMC immunoreactive and POMC mRNA-containing cells are found only in the AL; thus there is a developmentally regulated change in the extent of post-translational processing. Intron-exon splice junction probes have been used in solution hybridization experiments to determine the amounts of POMC, mRNA, and POMC primary transcript present during development; these data show that the ratio of primary transcript to mRNA is much higher during prenatal ages than in the adult. Finally, the reverse hemolytic plaque assay has been used to examine the ontogeny of functional receptors on differentiating POMC cells; these experiments have shown that CRH and glucocorticoid (GR) receptors are present in AL POMC cells shortly after their differentiation and that IL POMC cells transiently express a functional GR receptor.

Growth factors in limb development. ROBERT A. KOSHER, WILLIAM M. KULYK, KAREN GREER, AND BARBARA J. RODGERS (Department of Anatomy, University of Connecticut Health Center, Farmington, CT).

The possible involvement of fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β) in the regulation of cytodifferentiation and pattern formation during limb development was studied. Either acidic or basic FGF impairs the accumulation of cartilage matrix by limb mesenchymal cells *in vitro.* The anti-chondrogenic effect of FGF is potentiated by TGF-β. In contrast, TGF-β alone is a very potent stimulator of cartilage matrix formation and cartilage-specific gene expression *in vitro.* In fact, TGF-β promotes the formation of cartilage matrix and cartilage-specific gene expression in low density cultures in which little or no chondrogenic differentiation normally occurs. We propose that FGF, perhaps in conjunction with TGF-β, promotes the formation of non-chondrogenic tissues in the limb periphery during development, whereas TGF-β promotes cartilage differentiation in the central core of the developing limb.

JH-like compounds and their implications for crustacean reproduction. HANS LAUFER (Department of Molecular and Cell Biology, University of Connecticut, and the Marine Biological Laboratory, Woods Hole, MA).

Juvenile Hormone III (JHIII) is a major morphogen since it regulates development and reproduction in most insects. Since crustacea resemble the insects, we investigated the possibility of crustacea possessing a juvenile hormone-like compound. We found methyl farnesoate (MF) in the circulatory system of *Libinia emarginata.* It is synthesised and secreted by the mandibular organ (MO) *in vitro.* The synthesis coincides with vitellogenesis in adult females. Implants of MOs from adults into non-reproductive juvenile females, initiate ovarian growth. These experiments suggest that MF may be a crustacean JH. The eyestalks are known to regulate reproduction in adult female crabs by a gonad inhibiting hormone (GIH). Eyestalk removal increases MF production by the MOs. Adding eyestalk extracts to cultured MOs inhibited MF synthesis. We have attempted to identify eyestalk gonad inhibiting (GIH) and gonad stimulating hormones (GSH). We have found that synthetic pigment dispersing hormone (PDH) inhibits MF synthesis in *Procambarus clarkii* and red pigment concentrating hormone (RPCH) stimulates MF synthesis by MOs *in vitro.* The regulation of reproduction in crustacea may be through direct action of hormones on the gonads or by indirect action of neuropeptides on the MO.

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The metabolism of methyl farnesoate in Libinia emarginata. KENNETH M. ALBRECHT AND HANS LAUFER (Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT, and The Marine Biological Laboratory, Woods Hole, MA).

The metabolism of methyl farnesoate (MF), the major circulating juvenile hormone-like (JH) compound in *Libinia emarginata,* was investigated using an *in vitro* radiochemical assay combined with normal and reverse phase HPLC. MF is not converted to JHIII by the mandibular organ or potential target tissues. In all tissues studied, the major extractable metabolite was found to be farnesoic acid. No conversion was seen in the hemolymph. These results indicate that MF is not converted to JHIII and raises the possibility that farnesoic acid is both a precusor and metabolite of MF.

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In vivo *and* in vitro *studies on the calcium-dependent basal level expression of the rat prolactin gene.* G. M. PRESTON AND B. A. WHITE (Department of Anatomy, University of Connecticut Health Center, Farmington, CT).

The rat prolactin (rPRL) gene is expressed in a cell-specific manner. We and others have shown that the high basal expression of the rPRL gene is dependent on Ca^{2+}. Culturing of rat pituitary tumor GH$_3$ cells in a low Ca^{2+}, serum-free media (SFM) results in a minimal level of rPRL gene expression. Subsequent addition of 0.4 mM CaCl$_2$ to cells in SFM produces a significant increase in rPRL mRNA levels. Importantly, this effect of Ca^{2+} is highly specific for the rPRL gene, is stable, and is independent of serum factors. In order to identify cis- and transacting factors responsible for the calcium-dependent expression of the rPRL gene, we have developed an *in vitro* transcription assay. Using GH$_3$ and HeLa cell nuclear extracts we have been able to show tissue-specific gene expression with a clone containing 423 bp of 5′-flanking DNA. However, nuclear extracts prepared from cells in SFM with and without calcium both support transcription *in vitro* to a similar level. Therefore, the mechanisms that promote the calcium-dependent basal level expression to the rPRL gene may require chromatin structure and have to be elucidated *in vivo.*

Posters

Molecular and structural analysis of sex-linked translocations of chorion genes in Bombyx mori. M. G. ALEXOPOULOU, B. SAKAGUCHI[1], N. SPOEREL[2], S. NELSON, S. ZETLAN[3], AND M. R. GOLDSMITH (University of Rhode Island, Kingston, RI, [1]Kyushu University, Fukuoka, Japan, [2]U. Conn. Health Center, Farmington, CT, [3]U. Wisconsin, Madison, WI).

The genes that encode the chorion proteins of *Bombyx mori* are organized within two clusters on chromosome 2. In an attempt to understand the function of the chorion genes we have studied two sex-linked translocation strains, r06 and r07, in which the proximal end of chromosome 2 is attached to chromosome W. We have analyzed both translocations in two different homozygous genetic backgrounds, the wild type and the Gr^B deletion. The Gr^B deletion lacks all late (Hc) and many middle (A, B) chorion genes, leading to the formation of a defective chorion and female sterility. Both translocations carry extra chorion genes of middle developmental specificity, but lack early and late genes. This has been shown by genomic DNA hybridizations using a series of probes representing all developmental chorion families. The translocated genes are expressed, as revealed by two-dimensional gel electrophoresis of mature chorions. Both translocations rescue some of the effects of the Gr^B mutation but lack an outer crust, which we attribute to the late chorion genes.

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Patterns of ionic currents about the developing cockroach oocyte. E. Bowdan and J. G. Kunkel (Dept. Zoology, U. Mass., Amherst, MA 01003).

Patterns of ionic currents entering and leaving the vitellogenic oocyte of the cockroach, *Blattella germanica*, were examined using a two dimensional vibrating probe (Jaffe & Nuccitelli, 1974, *J. Cell Biol.* **63**: 614–628). The current vectors of small (<0.8 mm) oocytes all point either into or out of the oocyte without any focus and without regard to the position of the germinal vesicle. At about 0.8 mm, ventral vectors are tightly focused inwards in the mid-third of the oocyte, the region of the germinal vesicle. Mid-dorsal vectors point outwards. As the oocyte increases in size outward vectors become uniformly distributed across the dorsal surface and ventral inward vectors become less tightly focused. At approximately 1.4 mm, the distribution of dorsal outward vectors becomes bimodal, the vectors at the center becoming smaller, insignificant, or even in. At about 2 mm (shortly before chorionation) current density is low and no well-defined pattern can be seen.

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Relationship among cytoskeletal organization, fast axonal transport, and growth rate of neurites in cultures of chick embryonic dorsal root ganglia. K. T. Bush, M. C. Kosciuk, H. Lee, and R. G. Nagele (Department of Biology, Rutgers University, Camden, NJ and Department of Pediatrics, UMDNJ-School of Osteopathic Medicine, Camden, NJ).

Chick embryonic dorsal root ganglia (DRG) were grown in medium containing either B,B′-iminodipropionitrile (IDPN) or cytochalasin D (CD). At intervals, cultures were pulse-labeled with H^3-leucine, and autoradiography was performed after embedding in epoxy. Grain counts were made on the distal third of individual neurites. IDPN inhibited neurite sprouting and elongation in a dose-dependent manner and caused segregation of MTs from NFs which, however, had no apparent effect on the rate of fast axonal transport. CD differed from IDPN in that it inhibited, in a dose-related fashion, the rate of fast axonal transport which was not accompanied by a conspicuous change in the cytoskeletal organization in neurites. Overall results suggest that (1) the rates and mechanisms of fast axonal transport and the growth of neurites are unrelated and (2) the functional integrity of the molecular machinery for fast axonal transport in neurites is somehow dependent on actin, but independent of the spatial organization of MTs and NFs.

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Cytoplasmic rearrangements and dorsal-ventral axis formation in Xenopus laevis. J. M. Denegre and M. V. Danilchik (Department of Biology, Wesleyan University, Middletown, CT 06457).

The dorsal-ventral axis of the *Xenopus* embryo is established early in development. We are interested in the role that two major cytoplasmic movements play in determining the dorsal-ventral axis. The first of these movements is a flow of cytoplasm from the egg's interior toward the presumptive dorsal marginal zone. It occurs during the first cell cycle, and is probably related to the rotation of the vegetal yolk mass (Vincent *et al.*, 1986, *Dev. Biol.*). The second cytoplasmic movement is a cleavage-associated cytoplasmic ingression in which a thick subcortical, peripheral layer of cytoplasm advances inward during furrow formation. We studied the details of these two movements by labelling peripheral yolk platelets with nile blue sulfate or trypan blue in normal and UV-irradiated eggs.

The role of calcium in neural tube formation. A. S. DeNittis, M. W. McConville, K. T. Bush, C. L. Reckord, R. G. Nagele, and H. Lee (Department of Biology, Rutgers University, Camden, NJ and Department of Pediatrics, UMDNJ-School of Osteopathic Medicine, Camden, NJ).

As part of our interest in the role of calcium in neural tube formation, we cultured pieces of the chick neural plate (0.5×0.5 mm^2) in Medium 199 with or without a chemical agent (10–50 μg/ml). Chemical agents used were (1) bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA), a calcium chelator, (2) N,N,N′,N′-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), a heavy metal chelator with low affinity for Ca^{2+} and Mg^{2+} and (3) ethylene glycol bis(β-amino ethylether N,N,N′,N′-tetraacetic acid) (EGTA), a calcium chelator. After 24–36 hours of incubation, explants were fixed and processed for microscopy. All control explants (including those grown in medium with TPEN) underwent morphogenesis to form tubular structures closely resembling the closed neural tube of early chick embryos. In contrast, over 50% of those grown in medium containing BAPTA or EGTA showed little or no signs of further morphogenesis. Adversely affected neuroepithelial cells had smoother apical surfaces and less conspicuous microfilament bundles than usual.

Supported by the NIH.

Mouse tooth germ beta-glucuronidase activity in vitro. F. J. Dye (Department of Biological & Environmental Sciences, Western Connecticut State University, Danbury, CT).

Mycophenolic acid (MPA), an oncolytic agent produced by *Penicillium stoloniferum*, inhibits the conversion of inosine monophosphate (IMP) to guanosine monophosphate (GMP). MPA severly inhibits growth and development of mouse molar tooth germs in organ culture, but the simultaneous presence of guanine prevents the effects of MPA, while hypoxanthine does not. These results suggest that tooth germs salvage guanine to GMP while hypoxanthine, salvaged to IMP, is ineffective because the MPA inhibition is on the pathway from IMP to GMP.

Mycophenolic acid glucuronide (MPAG), a metabolite of MPA, does not enter cells and, therefore, does not exhibit inhibitory effects. Neither tooth germs nor the organ culture medium used exhibit sufficient beta-glucuronidase activity to free MPA from MPAG and cause inhibition. However, beta-glucuronidase incorporated into the medium, with and without (surprisingly!) MPAG, inhibited tooth germ growth and development.

In vitro *culture of avian scale skin with* de novo *synthesis of β-keratin.* Cynthia J. Fisher (Department of Biology, University of South Carolina, Columbia, SC 29208).

In vivo, glucocorticoids indirectly inhibit avian scale morphogenesis and beta keratin synthesis, probably by preventing placode formation (Fisher *et al.*, 1984). Investigation of direct steroid effects on skin differentiation require an *in vitro* model. In our system, avian skin explants are cultured on Nucleopore rafts floating at an air-liquid interface using DMEM plus 10% fetal bovine serum (FBS). Under these conditions, anterior metatarsal skin explants (AMS-Ex) from 7–9 day embryos developed poorly; 10–12 day explants formed shallow scales; and 13–18 day explants developed normal scutate scales. *De novo* synthesis of a beta stratum in 13–15 day AMS-Ex cultured for 7–13 days was confirmed by indirect immunofluorescent analysis for beta keratin. Two-D PAGE analysis indicated that the 7 beta keratins occurring in

Controls also occurred in 14-day AMS-Ex cultured for 7 days. Without FBS, 7–14 day AMS-Ex did not develop, whereas 15–18 day AMS-Ex were normal, suggesting that humoral factors are necessary for scale differentiation. On defined, low-steroid media 13–14 day AMS-Ex did not differentiate; the dermis was thick and hyperplastic.

Research supported by Research Corp.

Analysis of the Gr^{16} *chorion mutation of the domestic silkmoth,* Bombyx mori. DENISE GAUTREAU, MARIANNA ALEXOPOULOU, MARIAN R. GOLDSMITH (Department of Zoology, University of Rhode Island, Kingston, RI).

The *Gr*^{16} mutation of the domestic silkmoth, *Bombyx mori,* affects chorion morphology and ultrastructure. The first signs of abnormality appear during early chorion formation (G. D. Mazur, pers. comm.). To determine the molecular basis of the mutation we compared mature chorion protein composition, the gross structure of chorion genes, and the kinetics of protein synthesis in the homozygous mutant, its corresponding wildtype, and the heterozygote. Two-dimensional protein patterns of mature chorions were identical, indicating that *Gr*^{16} contained all major chorion components. Similarly, restriction patterns of the early chorion genes analyzed by Southern blot hybridization of genomic DNA revealed no differences, suggesting that the mutation lacked gross chromosomal defects. However, two pairs of early proteins showed reciprocal differences in relative labeling intensities: one pair labeled more intensely in the wild type, while the other was more prominent in *Gr*^{16}. These two pairs of proteins had similar molecular weights but different pKI's, which suggested that they were related. Preliminary labeling studies indicated that the mutation may affect post-translational modification of the proteins in question.

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Evidence of a tubular system from the plasmalemma in the egg cortex. N. H. HART, AND J. S. WOLENSKI (Dept. of Biological Sciences, Rutgers Univ., New Brunswick, NJ).

Studies with conventional transmission electron microscopy and freeze fracture suggest the presence of two tubular membranous systems in the unactivated egg cortex of the zebrafish, *Brachydanio rerio.* A cortical endoplasmic reticulum (CER) forms a complex labyrinth that appears to extend throughout the cortex and make close associations with the cortical granules. At the level of the plasma membrane, profiles of the CER are separated from the plasma membrane by a gap of 5–10 nm. A fuzzy, lightly staining material fills the circular and longitudinal profiles of this system. We have also observed membrane-limited tubular profiles in the cytoplasm that are devoid of substance and show a striking resemblance to invaginations of the plasmalemma. These profiles measure about 50–60 nm in diameter and are occasionally found in the vicinity of cortical granules. To determine if there was confluence between the egg plasma membrane invaginations and these tubular profiles of the cortex, we attempted to label the latter by exposing fixed eggs to either cationized ferritin, ruthenium red, or lanthanum nitrate. Cationized ferritin has been resolved in membrane profiles beneath the plasma membrane.

Ectopic Antennapedia *legs of* Drosophila melanogaster *reveal complete homology of positional information in antenna and second leg.* JOHN L. HAYNIE (Biology Department, SUNY College, Geneseo, NY).

In flies of the genotype Pc^{1} Antp^{73B}/Antp^{Ns} M(3)i^{55}, antenna and surrounding regions of the head capsule are transformed into mesothoracic leg; all recognizable cuticular structures of the ventral mesothorax normally derived from the second leg disc are found ectopically. A description of structure replacement and the implied homologies of positional information are given. The boundary of the affected region of the head corresponds to a weak clonal restriction detected in clonal analysis experiments. Orientation of the ectopic leg structures is modified, presumably due to the absence of more-anterior segments. Head segmentation and evolution of the insect segment are discussed.

An in vitro *bioassay for the regulation of methyl farnesoate synthesis in disaggregated mandibular organ cells from* Libinia emarginata *and the effect of serotonin.* E. HOMOLA, M. LANDAU, AND H. LAUFER (The University of Connecticut, Storrs, and the Marine Biological Laboratory, Woods Hole, MA).

Mandibular organ (MO) cells from *Libinia emarginata* were disaggregated using 0.25% collagenase. The rate of methyl farnesoate synthesis by the dissociated cells was determined by radiochemical assay to be linear for a two-hour period. Six pairs of MOs provided enough cells for 12 replicates; the variation among replicates was less than 10%. Serotonin significantly inhibited methyl farnesoate synthesis in a dose-dependent manner at physiological concentrations during a two-hour culture period. At 10^{-8} M, serotonin inhibited methyl farnesoate synthesis by 35% ($P > 0.0001$). Since the central nervous system is known to regulate methyl farnesoate synthesis *in vivo,* the results suggest that serotonin may function as a neuroregulator of methyl farnesoate synthesis in *Libinia emarginata*

Supported in part by grants from The Sea Grant College Program (NOAA-NA85AA-D-SGr 101-84-86), USDA (85-CRCR-1-1839), the Lady Davis Trust, and a Senior National Research Service Award from NIH.

p2-5: a gene whose expression is reduced following induction by retinoic acid of differentiation in F9 teratocarcinoma cells. BETSY HOSLER, GREGORY LAROSA, JOSEPH GRIPPO, AND LORRAINE GUDAS (Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, and Dana Farber Cancer Institute, Boston, MA).

Teratocarcinomas are tumors that arise from germ line tissues, and can contain a mixture of differentiated cell types, as well as uncommitted stem cells. Embryonal carcinoma (EC) cells, derived from these stem cells, are studied as a model of mouse development. The F9 line of EC cells grows in culture as stem cells, and differentiates following addition of retinoic acid (RA). The laboratory of Dr. Lorraine Gudas is investigating the molecular basis of this F9 cell response to RA.

Several cDNA clones differentially expressed in F9 cells were isolated by Dr. LaRosa. The amount of transcript for one of these, p2-5, rapidly decreases following RA addition. Using nuclear run on assays, this decrease was shown to occur at the level of transcription. Computer analysis of the DNA sequence of p2-5 indicates that it is not strongly homologous to any sequence presently in GenBank. An open reading frame has been identified which would code for a 20 kD protein. Both the mechanism by which RA decreases the transcription of the p2-5 gene, and the function of its protein are targets for future work.

Epithelial movement during Rana pipiens *gastrulation.* J. LEBLANC, M. YODER, AND I. BRICK (The College of Staten Island, CUNY and New York University).

During amphibian gastrulation, morphogenetic movements result in the primitive body plan. During this embryonic period, epithelial migration is one of the movements that function in establishing the definitive gastrula anatomy. In this study, cellular interactions at the lip areas of the blastopore in *Rana pipiens* embryos at successive stages during gastrulation have been investigated by transmission electron microscopy (TEM). Examination of lateral blastoporal lip areas of *Rana pipiens* embryos by TEM has indicated a number of points on the lip surface with elongated flask-shaped cells. The distal ends of these cells are constricted, often with the lip surface exhibiting an indentation. The investigators are currently planning high resolution video recording of developing lip areas in *Rana pipiens* embryos to correlate TEM images of the lip surface with observations in the living organism.

Microfilaments of developing chick neuroepithelial cells. F. J. LYNCH, A. S. DENITTIS, M. W. MCCONVILLE, K. T. BUSH, R. G. NAGELE, M. J. GOLDENTHAL, AND H. LEE (Department of Biology, Rutgers University, Camden, NJ and Department of Pediatrics, UMDNJ-School of Osteopathic Medicine, Camden, NJ).

In developing chick neuroepithelial cells, microfilaments are often organized into discrete bundles in their apices and are most prominent in regions where bending of the neuroepithelium occurs during neural tube formation. These microfilaments contain motility-related proteins (*e.g.*, actin and myosin), and their contractile activity is greatly affected by chemical agents (*e.g.*, local anesthetics and calcium agonists) known to alter intracellular calcium ion levels. Morphometric studies show that bending of the neuroepithelium and closure of the neural tube are driven by microfilament-dependent apical constriction of neuroepithelial cells. High-resolution transmission electron microscopy shows the presence of alternating dark and light areas (resembling those of skeletal muscle) along the length of microfilament bundles. *In situ* hybridization reveals that messenger RNA for actin is often most concentrated in apical regions of developing neuroepithelial cells shortly before bending of the neuroepithelium.

Supported by the NIH.

Expression of chorion-Adh promoter fusions in Drosophila. C. P. ROMANO AND F. C. KAFATOS (Harvard Biological Labs, Cambridge, MA).

During *Drosophila* oogenesis, the follicle cells express and amplify the chorion (eggshell) genes with temporal, tissue, and sex specificity. To identify *cis*- and *trans*-regulators of chorion gene expression, we fused the late s15 chorion gene promoter to the alcohol dehydrogenase structural gene and examined fusion gene expression in Adh null flies. As selections for and against Adh activity are well established in *Drosophila*, such fusions may permit the direct selection of mutations affecting chorion gene expression. The s15-Adh fusion genes are expressed with the same temporal, tissue, and sex specificity as the endogenous s15 gene, indicating that the 5′ flanking and promoter regions are sufficient to confer such specificity. However, steady state s15-Adh fusion gene mRNA levels are [illegible] lower than the levels produced by "marked" s15 gene constructs containing the s15 structural gene and 3′ flank. Actinomycin D experiments show that the Adh and "marked" s15 mRNAs have similar stabilities [illegible] indicate that the observed difference in steady state levels may be due to an element located 3′ to the s15 cap site which increases the rate of s15 transcription. Additional transformation as well as nuclear run-on experiments are underway to determine the basis of this quantitative effect.

The one-cell and early two-cell stage in preimplantation mouse embryo is temperature sensitive. A. W. SEITZ (Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA 01003).

When one-cell mouse embryos are cultured at 35°C, they remain viable and develop normally to blastocysts. The development of these embryos is delayed when compared to that of *in vivo* development and *in vitro* development at 37°C. While blastocysts are found on the fourth day *in vivo*, and on the fifth day when incubated at 37°C, they are not seen until the sixth day when cultured at 35°C. The transition from one cell to two cell is on schedule. The delay begins at the two-cell stage, and extends until the eight-cell stage. The transition from eight-cell and morula to blastocyst is comparable to those cultured at 37°C. When embryos were taken at the two-cell stage and cultured at 35°C, this marked delay was not seen. The sensitivity of the preimplantation mouse embryos to a lower temperature is confined to the period spanning the late one-cell and early two-cell stage. One of the most important processes that is specific to this period is the activation of the zygotic genome. It is possible that the processes involved in the transcription of the early genes are more temperature sensitive than other processes.

Extraembryonic epithelia can promote osteogenesis in maxillary mesenchyme of the embryonic chick. M. S. TYLER (Department of Zoology, University of Maine, Orono, ME).

Maxillary mesenchyme of the embryonic chick forms membrane bone, but requires the presence of an epithelium during early development in order to do so. The ability of extraembryonic epithelia from the chorioallantoic membrane to promote this osteogenesis was tested. Maxillary mesenchyme (HH stages 19–21; Hamburger and Hamilton, 1951) separated from its epithelium and grown as a graft on the chorioallantoic membranes of host chick embryos did not form membrane bone. However, when recombined directly with chorioallantoic endoderm or chorioallantoic ectoderm and then grafted, maxillary mesenchyme did form membrane bone in 87.5% and 70% of cases, respectively. This is the first report of an extraembryonic epithelium eliciting an osteogenic response from membrane bone-forming mesenchyme and indicates that the germ-layer origin of an epithelium does not necessarily restrict its potential to promote membrane bone formation.

Supported by research grant R01 DE04859 from the National Institute of Dental Research, of the National Institutes of Health.

Expression of Rhizobium meliloti *nod genes induced by exudate from mutants of white sweetclover.* LINDA J. UTRUP AND JOANNA H. NORRIS (Department of Microbiology and Department of Botany, University of Rhode Island, Kingston, RI).

The symbiotic association of *Rhizobium meliloti*, a soil bacterium, and *Melilotis alba*, white sweetclover, results in the formation of nitrogen-fixing nodules. Genes involved in the early stages of nodule development include the *nod*ABC operon and *nod*D genes of *Rhizobium* and nodule-specific plant genes. A flavone compound released by the plant, along with the constitutive *nod*D product, is required for induction of the *nod*ABC operon. To investigate this induction, exudate from ten non-nodulating *M. alba* mutant strains, generously provided by Dr. Tom LaRue, was analyzed. By utilizing a strain of *R. meliloti* containing a *nod*C-*lac*Z fusion, generously provided by Dr. Sharon Long, (Mulligan and Long, 1985, *PNAS* **82**: 6609–6613) and measuring β-galactosidase activity, induction of the *nod*ABC operon was determined. Results indicate that most of the inducer was present in the seed exudate collected after the first 24 hours. Seed and root exudate collected from the ten *M. alba* mutants induced expression of the *nod*ABC operon in *R. meliloti.*

Specific mRNA associated with cortex of giant silkmoth. C. WATSON, B. JARNOT, AND S. J. BERRY (Department of Biology, Wesleyan University, Middletown, CT 06457).

Specific binding of radioactive polyuridylic acid by sections of developing oocytes of *Hyalophora cecropia* indicates that the cortex may serve as a depot for maternal mRNA synthesized in the nurse cells. A cDNA library has been constructed from mature egg RNA of this species. Differential screen of the library using cDNA made against mRNAs from RNAs from the yolky cytoplasm and the cortical cytoplasm resulted in several clones that hybridized to a higher degree to the probes from the cortical fraction. *In situ* hybridization using one of these clones (Ec4b) shows preferential hybridization to the cortical cytoplasm. Analyses of the nucleotide and amino acid sequences reveal similarity to lepidopteran chorion genes and a lesser but convincing similarity to vertebrate cytokeratins. Aspects of the structure of the protein encoded by this mRNA suggest that it is a structural component necessary for the formation of the blastoderm of the embryo. The association of this maternal mRNA with the cytoskeleton presents interesting possibility of a mechanism for the spatial distribution of determinative elements in eggs.

Calcium binding proteins in an insect oocyte. Y. ZHANG[1] AND J. G. KUNKEL (Dept. Zoology, U. Mass., Amherst, MA 01003).

Cellular processes are regulated by second messengers, among which Ca^{2+} has been shown to act through binding to a family of proteins, the prototype of which is calmodulin (CA). CA plays a role in receptor-mediated endocytosis and it may facilitate vitellogenin (Vg) uptake. We also speculate that CA is involved in the breakdown of yolk granules during embryonic development. Both vitellin (Vt) and a putative CA have been purified from *Blattella germanica* ootheca. The putative CA bound ^{45}Ca and cross-reacted with anti-bovine brain CA. Binding of Ca^{2+} to Vt in both native and denatured states was measured at pH 6.7 using ^{45}Ca-chelex partitioning and ^{45}Ca autoradiography. The 50 kD Vt subunit still bound Ca^{2+} even after Vt was treated with protein phosphatase. Thus, the phosphate group is not necessary for binding. Antibody to *B. germanica* CA is being prepared. We wish to examine whether the calcium binding property of Vt is actually due to the conjugation of Vt with CA. In addition we plan to localize CA within the developing oocyte and embryo of *B. germanica.*

Supported by NSF grant DCB-851781.

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[1] Winner, Best Student Presentation Award (poster).

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Induction of Swimming in *Stomphia* (Anthozoa: Actiniaria) by Imbricatine, a Metabolite of the Asteroid *Dermasterias imbricata*

J. K. ELLIOTT[1,*], D. M. ROSS[1,†], C. PATHIRANA[2], S. MIAO[2], R. J. ANDERSEN[2,‡], P. SINGER[3], W. C. M. C. KOKKE[1,3,§], AND W. A. AYER[3]

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Abstract. Imbricatine, a benzyltetrahydroisoquinoline alkaloid released by *Dermasterias imbricata*, has been tested for its ability to elicit escape responses in prey of the asteroid. Bioassays demonstrated that imbricatine is very effective at causing the detachment and swimming response in *Stomphia coccinea*, but is less effective at eliciting the same response in *S. didemon*. Two fragments of imbricatine, the benzyltetrahydroisoquinoline and 3-methyl histidine portions, were relatively inactive. Imbricatine did not elicit detachment behavior in either *Urticina* (= *Tealia*) *piscivora* or *Epiactis lisbethae*. The lack of comparable biochemical data for other escape and avoidance behaviors precludes any firm explanations of general patterns of chemical recognition of predators and non-predators by marine invertebrates.

Introduction

Many marine organisms exhibit escape and avoidance responses to chemicals released by their predators. The best documented of these behaviors are the responses of a wide variety of invertebrate prey species to chemicals secreted by predatory asteroids and mollusks (Feder, 1967; Edmunds, 1974; Mackie and Grant, 1974; Mayo and Mackie, 1976; Phillips, 1975, 1977, 1978; Sloan, 1980; Watanabe, 1983; Harvey *et al.*, 1987). Despite the large number of studies that have demonstrated chemical recognition of predators by their prey, "relatively little information is available concerning the nature of the substances responsible" (Mackie and Grant, 1974). To understand the chemical basis for these behaviors both qualitative and quantitive information is needed. However, the chemical structure of the active substances and the quantities required to elicit the behaviors have been elucidated in only a few cases (Turner *et al.*, 1971; Harvey, 1985; Harvey *et al.*, 1987). This makes it difficult to answer many general questions about the nature of chemical recognition in these interactions.

One of the best studied and most dramatic escape responses of a marine invertebrate is the detachment and swimming behavior of *Stomphia* (*S. coccinea* and *S. didemon*) in response to certain species of asteroids and the nudibranch *Aeolidia papillosa*. This escape response has interested scientists since it was first described by Yentsch and Pierce (1955) and has resulted in a wide variety of studies on different aspects of the interaction: behavioral (Robson, 1961a; Ross, 1965a, b, 1967a, b, 1979; Ross and Sutton, 1964a, 1967a, b), neurophysiological (Hoyle, 1960; Robson, 1961b; Ross and Sutton, 1964b; Lawn, 1976, 1980), morphological (Sund, 1958; Rob-

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[†] Deceased. D. M. Ross initiated this project and sustained interest in the work for over 15 years. This paper is dedicated to him.

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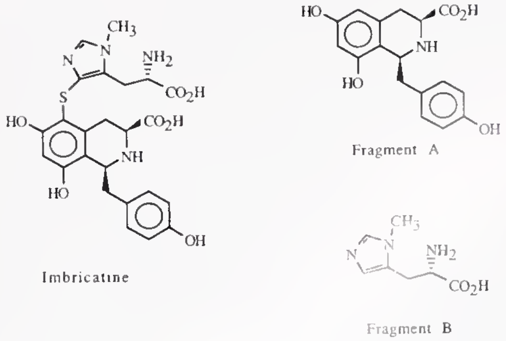

Imbricatine

Fragment A

Fragment B

Figure 1. Chemical structures of imbricatine, Fragment A and Fragment B.

son, 1963; Peteya, 1976), ecological (Mauzey *et al.*, 1968; Dalby *et al.*, 1988), and chemical. The first work towards identifying the chemical(s) that elicits the response in *S. coccinea* was by Ward (1965), whose quantitative analyses of the partially purified substance isolated from the asteroid *Dermasterias imbricata* seemed to indicate that the active compound was an amino polysaccharide. However, most of the chemicals previously isolated from asteroids that had been found to elicit escape behaviors were saponins, and Mackie (1970) emphasized that Ward's material was not pure and may have contained asterosaponins.

Several years ago we set out to determine the complete chemical structure of the swimming substance present in the coelomic fluid of *D. imbricata* that elicits swimming in *S. coccinea* (Ayer *et al.*, 1973; Singer, 1975). A bioassay-guided fractionation of the methanol extracts of cut up specimens of *D. imbricata* yielded a single pure substance, which we named imbricatine, that was capable of eliciting pedal disk detachment and swimming in *S. coccinea*. Further structural studies (Pathirana and Andersen, 1986) showed that imbricatine was not an amino polysaccharide or an asterosaponin, but a benzyltetrahydroisoquinoline alkaloid (Fig. 1). Tetrahydroisoquinoline alkaloids are well known from terrestrial plants, however, imbricatine represents the first example of this family of alkaloids to be isolated from an animal source. The 3-methylthiohistidine substituent, the C3 carboxyl substituent, and the C6/C8 hydroxylation pattern on the tetrahydroisoquinoline ring represent structural features not previously encountered in this family of alkaloids. The function and metabolic origin of imbricatine is unknown.

Our work on the chemical structure of imbricatine provided pure samples of this chemical as well as pure samples of the benzyltetrahydroisoquinoline (Fragment A) and the 3-methyl histidine (Fragment B) portions of imbricatine (Fig. 1). Fragments A and B were obtained by Raney Nickel catalyzed hydrogenation (Pathirana and Andersen, 1986). The major objectives of this paper are: (1) to demonstrate that imbricatine does elicit the swimming response in *Stomphia coccinea* and *S. didemon*, and is effective at very low concentrations, and (2) to examine some of the implications of our bioassays with this chemical and two of its fragments to some general questions concerning chemical recognition in marine invertebrates.

Materials and Methods

Biological assays of imbricatine and Fragments A and B were done with specimens of *S. coccinea* collected by dredging in San Juan Channel, Washington. The animals were held at the Bamfield Marine Station, Bamfield, British Columbia, in running seawater (11°C) trays. Of 10 sea anemones collected, only 7 showed consistent pedal disk detachment and swimming responses to contact with the asteroid *D. imbricata*. These seven anemones were allowed to attach in small glass petri dishes. Tests for the effectiveness of the chemicals (in comparison to a seawater control) and the concentrations needed to elicit the response were conducted as follows. A petri dish with an attached sea anemone was taken out of the water, the remaining water was poured out of the dish, and then 50 μl of test solution was placed onto the oral disk of the sea anemone. The following behaviors of the sea anemone in response to the chemical were recorded: (1) retraction of the tentacles and closure of the oral disk, (2) elongation of the column, (3) opening of the oral disk and inflation of the tentacles, (4) flexions of the column, and (5) detachment of the pedal disk from the substratum and flexions of the column (swimming). If the anemone did not detach its pedal disk in response to the test solution, 50 μl of a crude extract of the aboral surface of *D. imbricata* (10 g aboral surface tissue in 100 ml filtered seawater) was then added to be certain that the animal was responsive. Trials in which animals did not respond to the crude extract were not used in the final data analysis. Tests with different chemicals at various concentrations were done in random order at approximately 48-hour intervals. The sea anemones were fed pieces of shrimp muscle after each third trial. Further trials were also done with the sea anemones *Stomphia didemon*, *Urticina piscivora*, and *Epiactis lisbethae* (Ross and Sutton, 1967; Elliott *et al.*, 1985) which are also known to detach in response to *D. imbricata*.

The chemicals tested were imbricatine, Fragments A and B, and a 1:1 combination of Fragments A and B. Solutions were prepared by dissolving solid forms of the chemicals in filtered seawater. Serial dilutions of imbricatine were done starting with a 2×10^{-3} M solution (1 mg/ml), resulting in a series of 7 solutions ranging in con-

Table I

Behavioral responses of Stomphia coccinea *to imbricatine, benzyltetrahydroisoquinoline (Fragment A), 3-methylhistidine (Fragment B), and a combination of Fragments A and B.*

Solution	Molarity	Number of trials	Behaviors elicited*				
			1	2	3	4	5
Imbricatine	2×10^{-3}	14	+	+	+	+	+
Imbricatine	2×10^{-4}	14	+	+	+	+	+
Imbricatine	2×10^{-5}	12	+	+	+	+	+
Imbricatine	2×10^{-6}	19	+	+	+	+	+
Imbricatine	2×10^{-7}	15	14	14	14	1	1
Imbricatine	2×10^{-8}	15	3	−	−	−	−
Imbricatine	2×10^{-9}	14	−	−	−	−	−
Imbricatine	2×10^{-10}	14	−	−	−	−	−
Fragment A	3×10^{-2}	6	+	+	+	+	1
Fragment A	3×10^{-3}	8	5	5	5	−	−
Fragment A	3×10^{-4}	10	−	−	−	−	−
Fragment B	3×10^{-3}	10	−	−	−	−	−
Fragments A + B	3×10^{-3}	9	4	4	4	−	−

* Refer to table caption for explanation of behaviors. (+) indicates that responses occurred in 100 percent of the trials, and (−) indicates no response. Number indicates how many successful responses out of the total number of trials.

The behaviors numbered in table as: (1) retraction of tentacles, (2) elongation of the column, (3) opening of the oral disk and inflation of the tentacles, (4) flexions of the column, and (5) detachment of the pedal disk and flexions of the column (swimming).

centration from 2×10^{-3} *M* to 2×10^{-10} *M*. Fragment A was initially assayed using a 3×10^{-2} *M* solution (1 mg/ml) and was then serially diluted to concentrations of 3×10^{-3} *M* and 3×10^{-4} *M*. Small amounts of Fragment B only allowed testing at a molarity of 3×10^{-3} *M*. A solution that was 3×10^{-3} *M* in both Fragment A and Fragment B was also tested.

Results

When a solution of imbricatine was placed onto the tentacles of a sea anemone it usually responded by going through the five stages of the swimming response outlined above (greater than 95% of trials). None of the sea anemones responded to filtered seawater control. During trials with high concentrations of imbricatine, all of the sea anemones detached and swam (Table I). Consistent responses were obtained with solutions down to a molarity of 2×10^{-6} *M*. The chemical was less effective at 2×10^{-7} *M* and there were no responses to solutions less than 2×10^{-8} *M*.

Fragments A and B were not as effective as the parent chemical (Table I). The anemones were less responsive, taking a longer time to react and not showing the complete behavioral response. At a high molarity, Fragment A elicited most of the behavioral response except detachment of the pedal disk and swimming. Of six trials with a 3×10^{-2} solution of Fragment A, only one of the sea anemones detached and swam. Five of the sea anemones retracted quickly in response to the chemical, elongated the column, and opened the oral disk but did not detach. The sea anemones did not respond to the chemical at lower molarities. Fragment B did not elicit a strong response from the anemones and most individuals did not react to the chemical. When Fragments A and B were combined, a response similar to that of Fragment A alone was elicited in some of the anemones.

Stomphia didemon also detached and swam in response to imbricatine. However, numerous trials revealed that some individuals would not consistently detach to even high concentrations of the chemical (2×10^{-3}), and it appears as if this species is not as sensitive to imbricatine as *S. coccinea*. The sea anemones *Urticina piscivora* and *Epiactis lisbethae* did not respond during trials (5 trials for each species) with imbricatine (2×10^{-3}). These sea anemones required physical contact with the seastar to elicit the detachment response.

Discussion

Studies examining the chemical basis of escape and avoidance behaviors of marine invertebrates to seastars have generally found that saponins are the reactive substances (Mackie and Grant, 1974; Harvey *et al.*, 1987). These results, in combination with the widespread occurrence of saponins in seastars (Burnell and ApSimon, 1983), have fostered the common perception that most of these behavioral responses are elicited by saponins

(Mackie and Grant, 1974; Burnell and ApSimon, 1983). However, Burnell and ApSimon (1983) and Mayo and Mackie (1976) reported that the active materials in some asteroids are not saponins. Our results also demonstrate that chemicals other than saponins are used by prey organisms to identify their predators. Thus, quantitative bioassays conducted with pure samples may reveal that other novel chemicals are responsible for eliciting particular escape and avoidance responses in other species.

The results of this study demonstrate that the *D. imbricata* metabolite, imbricatine, is extremely effective at eliciting the detachment and swimming behavior of *S. coccinea*. As little as 50 nanograms of the chemical (50 μL of a 2×10^{-6} *M* solution) was sufficient to induce the full behavioral response in 100% of the trials. Few other studies have measured the concentrations of the chemicals used in bioassays, but imbricatine is effective at least an order of magnitude lower than that reported for saponins isolated from seastars which elicit escape behaviors in the gastropod *Buccinum undatum* (Mackie *et al.*, 1968; Harvey *et al.*, 1987). The benzyltetrahydroisoquinoline portion of imbricatine (Fragment A) was at least four orders of magnitude less effective at eliciting the response than the whole chemical. This fragment appears to be the more reactive part of the chemical, since the 3-methylhistidine portion (Fragment B) was not effective at the relatively high concentrations tested. Previous studies have demonstrated that two or three chemicals may act synergistically to elicit escape behaviors (Mayo and Mackie, 1976), but a 1:1 mixture of imbricatine Fragments A and B was relatively inactive, ruling out any synergistic interaction of these two unattached fragments. The very low concentrations of imbricatine needed to elicit the response and the stringent structural requirements for biological activity suggest the presence of a selective chemoreceptor in *S. coccinea*.

Prey escape and avoidance behaviors are usually specific to only natural predators, but they may also be elicited by non-predators (Sloan, 1980). It is generally assumed that non-predators cause reactions because they are biochemically similar to the predators, either through taxonomic relatedness or similarity in diet (Mauzey *et al.*, 1968; Margolin, 1976; Dalby *et al.*, 1988). For example, *S. coccinea* and *S. didemon* have strong reactions to three species of asteroids in the order Valvatida but display weak or no reactions to at least 14 other species of asteroids (Ward, 1965; Dalby *et al.*, 1988). Only two of the asteroids that cause the response (*D. imbricata* and *Hippasteria spinosa*) are known to feed on these anemones, and the third species *Asterina* (= *Patiria*) *miniata* does not feed on cnidarians. This indirect evidence suggests that the three closely related asteroids contain similar quantities of the same chemical, and other asteroids that cause weak responses have either smaller quantities of this chemical or similar chemicals that are less reactive. However, as in most studies, there is no direct evidence to confirm these inferences. It is unknown if asteroids that elicit a particular response contain the same chemicals or if the prey use the same chemicals, or quantities of them, to recognize each asteroid species (Mackie and Grant, 1974).

Some studies have shown that prey discriminate among asteroids by detecting different chemicals released by each species. Mackie and Grant (1974) report that the major steroid glycoside present in the seastar *Marthasterias glacialis* is not present in other asteroid species that cause the same behavioral responses in some marine invertebrates. Also, preliminary structural analyses in our laboratory suggest that the active chemical in the asteroid *Hippasteria spinosa* that causes *Stomphia* spp. to swim is not similar to imbricatine (Andersen, unpub. obs.). Thus, some prey appear to have different receptors for different species of asteroids, and may possess more finely tuned recognition systems than has been previously assumed. We are presently trying to isolate and identify the chemicals from other asteroids which elicit swimming in *Stomphia* spp. to better answer this question.

To increase the ability to discriminate among predators and non-predators, it is likely that many marine invertebrates cue on more than one particular chemical or other type of stimuli. Harvey *et al.* (1987) found that purified saponins were not as effective at eliciting an escape behavior in *Buccinum undatum* as natural seastar "scented" water, which suggested that the prey may also be responding to other substances released by the asteroid. Our bioassays of the different isolated fractions from *D. imbricata* indicate that imbricatine is the only chemical required to elicit a complete response in *S. coccinea*. But, *S. didemon* was relatively less sensitive to imbricatine. The variation in the responses of different prey organisms to the same species of asteroid is likely due to differences in the type or sensitivity of the chemoreceptors of the prey (Mackie and Grant, 1974). Mackie and Grant (1974) have reported that the same saponins (steroid glycosides) from *M. glacialis* induce behaviors in many different species of prey. Similarly, the reaction of *S. didemon* to imbricatine demonstrates that it has the same or similar type of receptor found in *S. coccinea*, but *S. didemon* may require either greater quantities of this stimulus or different types of cues. *Stomphia didemon* detaches and swims most consistently when it is touched by asteroids. The anemone may also recognize particular cell surface properties of the asteroid. Imbricatine did not elicit escape responses in *Tealia piscivora* or *Epiactis lisbethae*, and these sea anemones also appear to require physical contact cues from *D. imbricata*. Thus, various prey species may cue on the same or different chemicals, or other characteristics of the same predator.

There is no chemical evidence to support the hypothesis that prey differentiate among seastars on the basis of the quantity of the substance released. Phillips (1978) has presented indirect evidence that the sea urchin *S. droebachiensis* may recognize the difference between foraging and non-foraging *Pycnopodia helianthoides* by cueing on the concentration of chemical(s) released from the asteroid when it is moving compared to when it is stationary. But, it is clear that direct evidence, through chemical studies of the substances involved, is needed to obtain a satisfactory answer to this question.

Our study has demonstrated that *S. coccinea* and *S. didemon* react to a specific chemical released from the asteroid *D. imbricata*. This has confirmed some early inferences by Ross (1966) and others that even though sea anemones are relatively simple animals they have highly evolved sensory systems and complex behavioral responses to very specific stimuli. Structural studies of imbricatine showed that it was not a saponin, which suggests that further studies of the substances that elicit escape behaviors in a wide variety of prey species may reveal that other novel chemicals are used for recognition. We have attempted to address some general questions concerning chemical recognition in marine invertebrates, but the lack of similar biochemical data for comparison precludes any firm explanations of the chemical basis for observed patterns of responses among prey to different species or physiological states of predators and non-predators. Research in this area has not advanced much in the last decade, and we re-emphasize the statement of Mauzey *et al.* (1968) that "the whole subject of avoidance responses of invertebrate animals can only be clarified finally by further, detailed biochemical studies."

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Ultrastructure of the Ovary and Oogenesis in the Ovoviviparous Ophiuroid *Ophiolepis paucispina* (Echinodermata)

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Abstract. *Ophiolepis paucispina* (Say) produces large yolky oocytes and has ovoviviparous reproduction. The ultrastructure of the somatic tissue layers of the ovarian wall and the germinal epithelium of *O. paucispina* is described. Oogenesis is continuous with oocytes at different stages of development interspersed in the ovary. Proliferation of oogonia and initial growth of the primary oocytes occur within a follicle. Thereafter, the oocytes are largely independent of the germinal layer, and oogenesis is solitary. The oocytes bulge outwards as they grow, forming a sac-like depression in the ovarian connective tissue, and are connected to the germinal layer by a short stalk. Subsequently, haemal fluid accumulates in the connective tissue adjacent to each vitellogenic oocyte. Although each oocyte is enveloped by haemal fluid, it is separated from the fluid by the inner basal lamina. Vitellogenesis involves an accumulation of yolk bodies that appear to be formed jointly by the endoplasmic reticulum and the Golgi complex. Endocytotic activity is prevalent throughout vitellogenesis, indicating incorporation of exogenous material by the oocytes. It is suggested that the genital haemal sinus serves as an intragonadal nutrient store and that yolk precursors, potentially a component of haemal fluid, may be sequestered by the oocytes. *O. paucispina* appears to have heterosynthetic yolk formation and the evidence for this mechanism of vitellogenesis in the Echinodermata is discussed.

Introduction

Although the ultrastructure of echinoderm oocytes is described in several investigations, the cellular events of oogenesis and the dynamics of yolk deposition are not well documented (reviews: Piatigorsky, 1975; Walker, 1982; Kanatani and Nagahama, 1983). Most studies of echinoderm oogenesis describe aspects of asteroid and echinoid oocyte development (Piatigorsky, 1975; Walker, 1982; Kanatani and Nagahama, 1983; Beijnink *et al.*, 1984c; Ferrand, 1984; Aisenshtadt and Vassetzky, 1986) and there is a recent investigation of holothuroid oogenesis (Smiley, 1988). For ophiuroids, there are two ultrastructural studies of oogenesis (Kessel, 1968; Byrne, 1988), and several thesis reports on the ultrastructure of the ovary and the oocytes (Patent, 1968; Davis, 1971; Bell, 1974; Tyler, 1976).

This study describes the ultrastructure of the ovary and oogenesis of *Ophiolepis paucispina* (Say), a small hermaphroditic ophiuroid (5–7 mm disc diameter) that broods its young in the genital bursae (Hendler, 1979). Like most brooding ophiuroids (Hendler, 1975, 1979; Strathmann and Strathmann, 1982), *O. paucispina* is ovoviviparous and produces a small number (2–20) of large oocytes (400 μm diameter). Embryonic development appears to be completely supported by the nutrient reserves present in the oocyte. In contrast to the viviparous ophiuroid *Amphipholis squamata* (Delle Chiaje), extra-embryonic nutrient reserves are not present in the bursal wall of *O. paucispina* (Fell, 1946; pers. obs.).

Recent interest in echinoderm oogenesis has focussed on oocyte nutrition (Walker, 1982; Voogt *et al.*, 1985). For many echinoderms, it appears that somatically derived nutrients are delivered to the ovary by coelomic transport and stored for oogenesis by the haemal system (Walker, 1982; Ferguson, 1982, 1984; Voogt *et al.*, 1985; Beijnink and Voogt, 1986; Byrne, 1988). The somatic synthesis of vitellogenin has been demonstrated biochemically for echinoids (Harrington and Ozaki, 1986;

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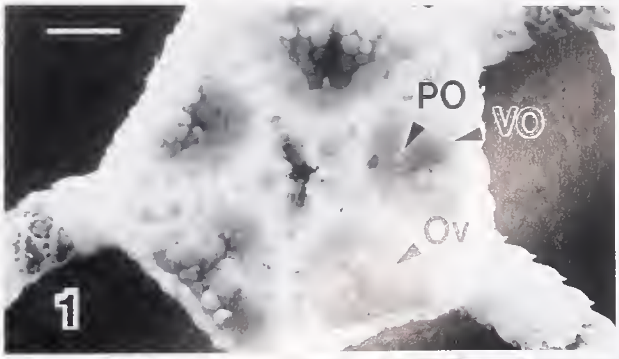
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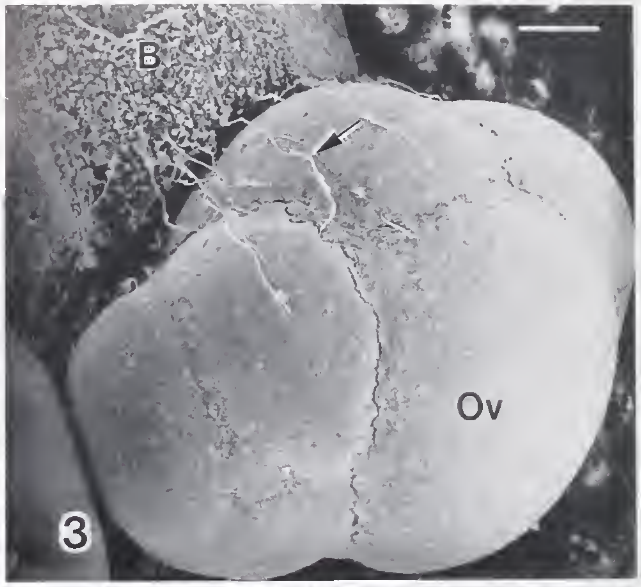
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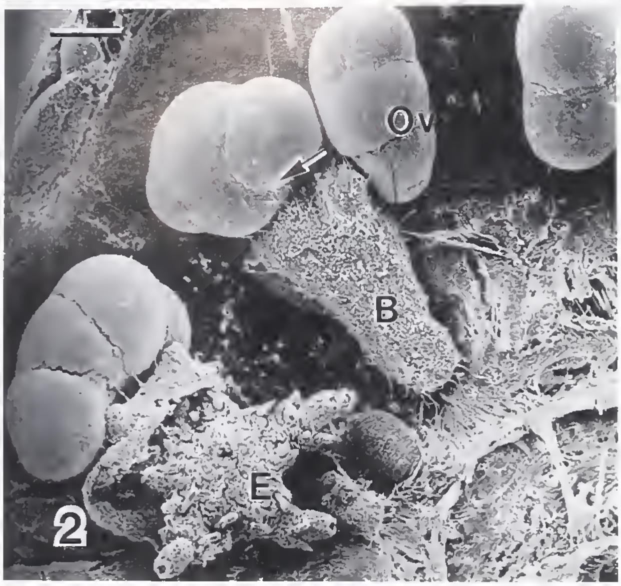
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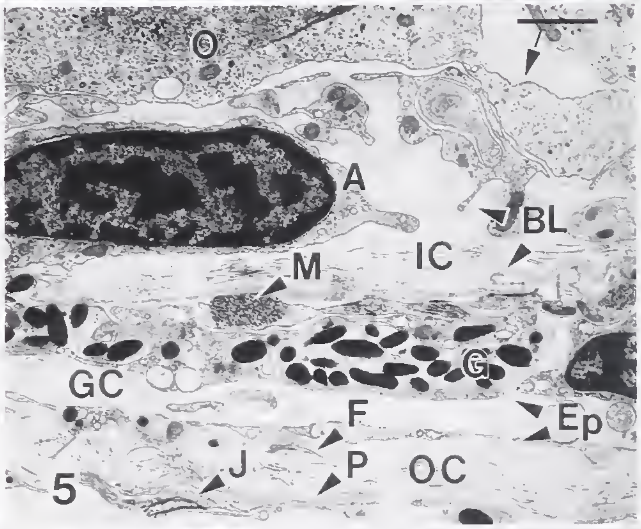
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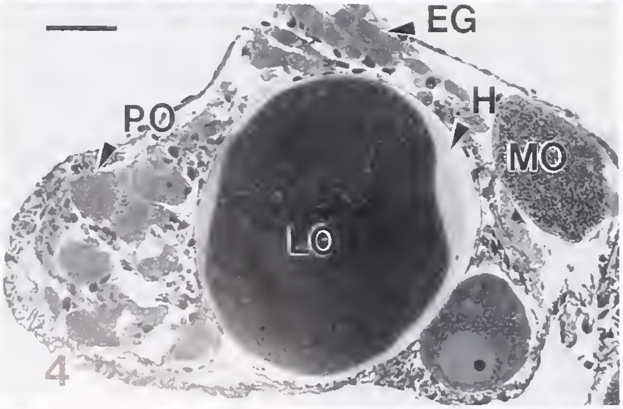
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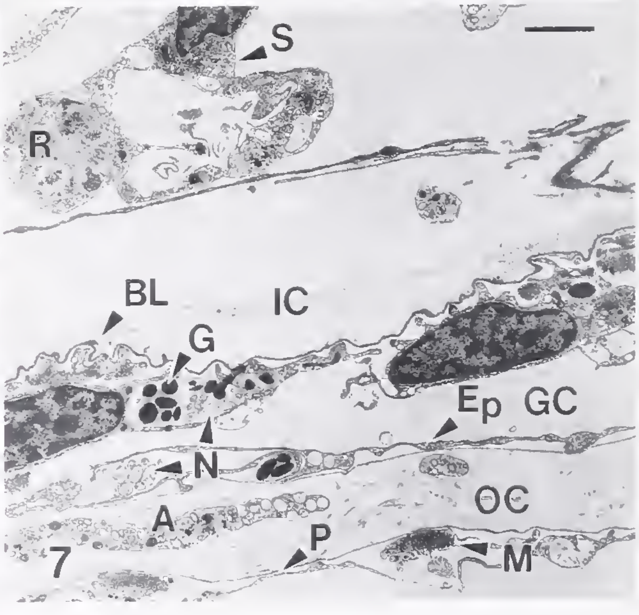
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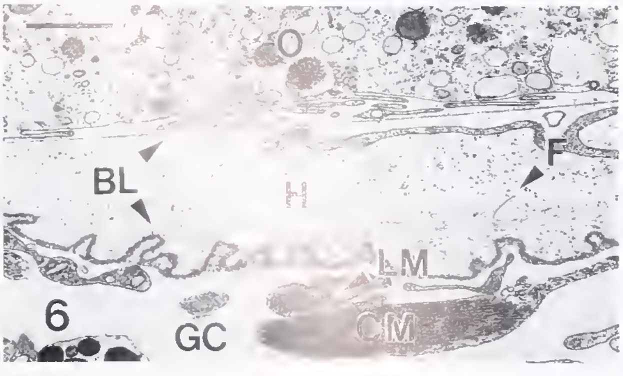
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Shyu *et al.*, 1986). As in other echinoderms (Walker, 1982), the genital haemal sinus of *O. paucispina* contains PAS + material and vitellogenesis involves the elaboration of PAS + yolk, suggesting the incorporation of haemal material by the oocytes (Byrne, 1988). Moreover, the presence of endocytosis by the oocytes indicates uptake of exogenous material (Byrne, 1988). In this investigation, the developmental sequence of oocyte growth in *O. paucispina* is documented from the early proliferative stage through the previtellogenic and vitellogenic stages. Emphasis is placed on the interaction between the germinal and the somatic cells of the germinal epithelium, the role of the genital haemal sinus, and on the vitellogenic mechanisms involved in the production of the yolky oocyte. This is the first such investigation for an ophiuroid and its significance is assessed with respect to current concepts of ovarian structure and oocyte development in echinoderms.

Materials and Methods

Specimens of *Ophiolepis paucispina* were collected at 0.5–1.0 m depth from clumps of the calcareous alga *Halimeda* from Conch Key, Florida (monthly, January through December, 1985), and from Southwater and Carrie Bow Cays on the Belize Barrier Reef (June, 1985, and April, 1986). Specimens for transmission electron microscopy were placed in primary fixative on a cold plate without primary anaesthesia. The ovaries were excised and placed in fresh fixative. Four fixation methods were employed, including phosphate buffered glutaraldehyde (Cloney and Florey, 1968), cacodylate buffered glutaraldehyde (Walker, 1979), and seawater buffered glutaraldehyde (Buckland-Nicks *et al.*, 1984). Osmication followed primary fixation using the same buffer (Cloney and Florey, 1968; Walker, 1979) or sodium bicarbonate (Eckelbarger, 1979; Buckland-Nicks *et al.*, 1984). Best fixation was obtained with the method of Buckland-Nicks *et al.* (1984) as follows: the ovaries were fixed in 2.5% glutaraldehyde in 0.45 μm millipore-filtered natural seawater for 1 h at room temperature. This was followed by a rinse in 2.5% sodium bicarbonate (pH 7.2) and secondary fixation in 2% osmium tetroxide in 1.25% sodium bicarbonate for 1 h at room temperature. Following fixation, the tissue was rinsed in distilled water, dehydrated in ethanol, transferred to propylene oxide, and embedded in Epon 812. The cacodylate method (Walker, 1979) yielded the best preservation of the cortical granules and the vitelline envelope. For light microscopy, 1-μm sections were stained with a mixture of methylene blue and Azur II. Thin sections were stained with 2% uranyl acetate followed by 2% lead citrate and viewed with a Zeiss EM9-S2 electron microscope.

Specimens for scanning electron microscopy were also fixed according to Buckland-Nicks *et al.* (1984), dehydrated in ethanol, transferred to acetone, and critical point dried. The dried specimens were mounted, sputter coated, and viewed with a Novascan 30 or Hitachi S-570 SEM.

Results

Structure of the ovary

The ovaries of *Ophiolepis paucispina* are attached to the ambulacral or interambulacral genital ossicles positioned on either side of the bursal slits and project into the perivisceral coelom (Figs. 1, 2). There are up to three ovaries per bursa ranging in length from 600–1000 μm. Suspensor cells connect the peritoneal lining of the bursa and the ovary wall (Figs. 2, 3). Reproduction is continuous with the ovaries containing oocytes at different stages of development throughout the year (Figs. 1, 4). Vitellogenic oocytes are yolky and pale pink in life, and at the

Figure 1. *Ophiolepis paucispina* with the aboral surface removed to show the ovaries (Ov) containing previtellogenic (PO) and vitellogenic (VO) oocytes. Scale = 1.0 mm.

Figure 2. Scanning electron micrograph (SEM) showing the ovaries (Ov), peritoneal suspensors (arrow), bursal wall (B), and an embryo (E) emerging from a torn bursa. Scale = 300 μm.

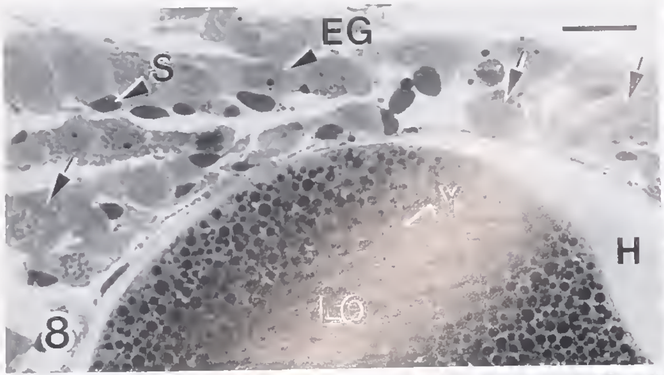
Figure 3. SEM of an ovary (Ov) and peritoneal suspensors (arrow). B, bursal wall. Scale = 100 μm.

Figure 4. Light microscopic (LM) section of an ovary with late (LO), mid- (MO), and previtellogenic (PO) oocytes. EG, early germinal cells, H, haemal fluid. Scale = 75 μm.

Figure 5. Transmission electron micrograph (TEM) of the ovary wall in cross section. The inner and outer sacs are separated by the genital coelom (GC). A, amoebocyte, BL, basal lamina, Ep, coelomic epithelium, F, collagen fibrils, G, granulated process, IC, inner connective tissue, J, cell junction, M, myoepithelial cell, OC, outer connective tissue, P, visceral peritoneum, Arrow, basal process of the primary oocyte (O). Scale = 2.0 μm.

Figure 6. Next to a vitellogenic oocyte (O) the inner connective tissue layer is filled with haemal fluid (H). The inner basal lamina (BL) is thin while the outer basal lamina is thick and plicated. CM circular muscle cell, F, fibril, GC, genital coelom, LM, longitudinal muscle cell. Scale = 1.5 μm.

Figure 7. The inner connective tissue (IC) is devoid of haemal fluid next to relict oocyte material (R). A, amoebocyte, BL, basal lamina, Ep, epithelium, G, granulated process, GC, genital coelom, M, muscle, N, nerves, OC, outer connective tissue, P, peritoneum, S, somatic cell. Scale = 3.0 μm.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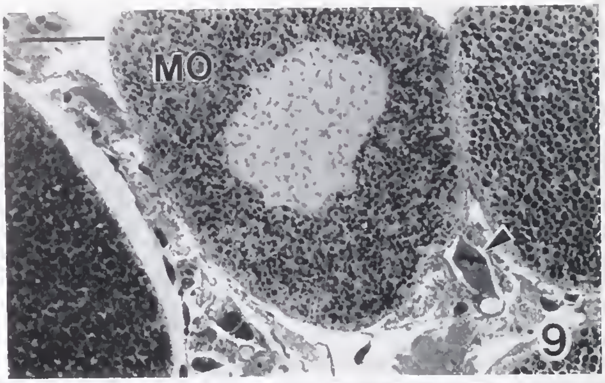
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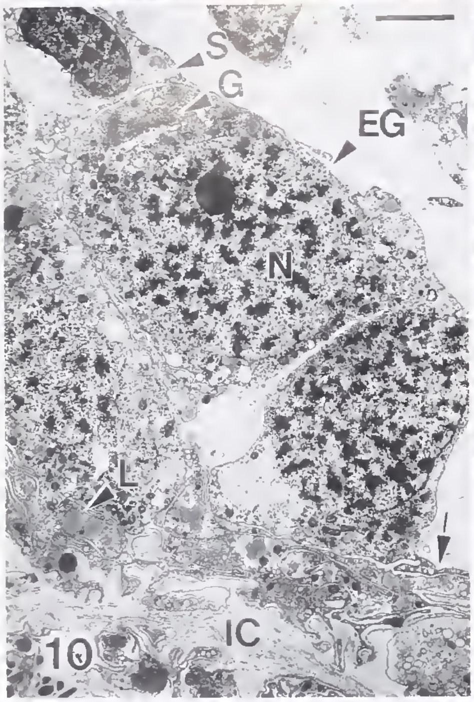
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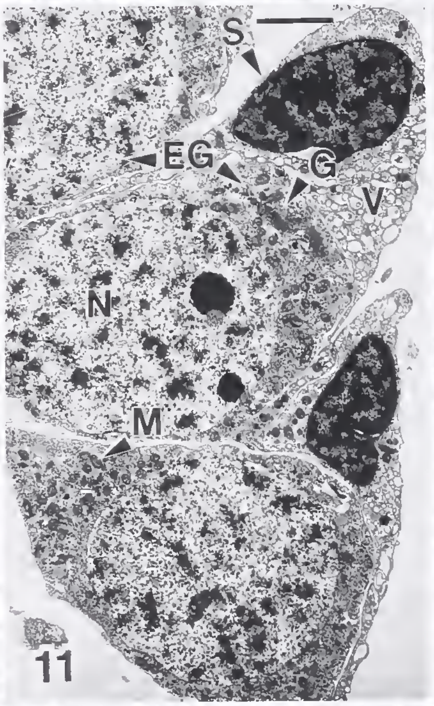
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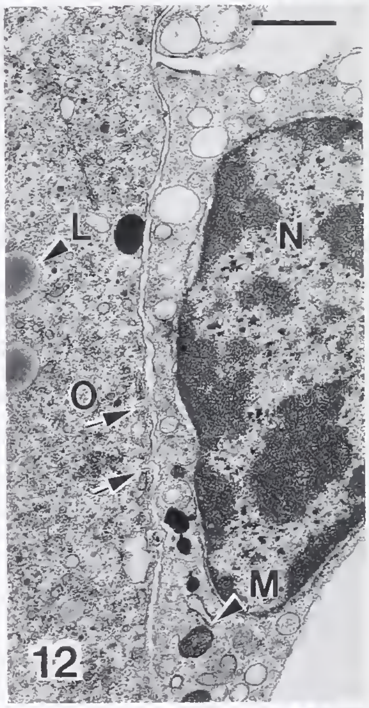
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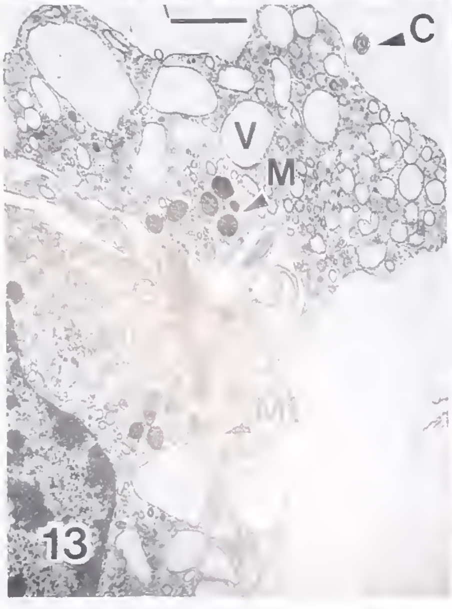
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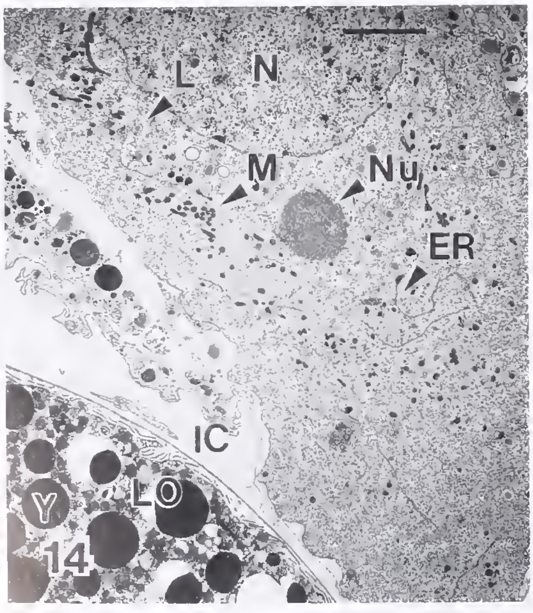
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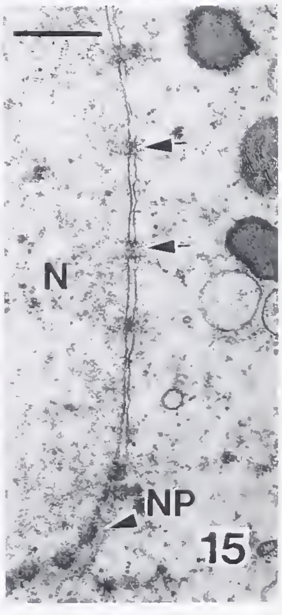
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end of oogenesis, are 400 μm in diameter (Hendler, 1979). Oviducts are absent and the oocytes are spawned into the bursal sac one to a few at a time by an unknown mechanism. Subsequent fertilization presumably occurs in the bursa. The embryos develop in the bursa leaving the adult at the two to three arm segment stage (Fig. 2).

The ovary consists of two parts, the inner and outer sac, which are separated by the genital coelom (Figs. 5, 7, 45). Each sac is composed of three layers of tissue. The outer sac consists of the visceral peritoneum, a central connective tissue layer, and the genital coelomic epithelium. The inner sac is composed of the internal coelomic epithelium, a connective tissue layer that contains the haemal sinus, and the germinal epithelium.

The outer covering of the ovary, the visceral peritoneum, is a thin layer of epithelial and myoepithelial cells, each containing an elongate nucleus, mitochondria, vesicles and lysosomes (Figs. 5, 7). Cell bodies of the peritoneal cells were rarely encountered and sparse cilia arising from the peritoneal layer were seen in the light microscopic sections. Small bundles of neurons containing dense-cored (60–120 mm diameter) and clear vesicles (60–140 nm diameter) run beside the myoepithelial cells and are covered by cytoplasmic extensions from these cells (Figs. 7, 35). Processes containing large electron-dense granules (500–1500 nm length) are also present in the peritoneum (Figs. 5, 7). In general, the ovary is well innervated with longitudinally orientated nerve tracts surrounded by epithelial cell processes. Zonulae adherentes and septate junctions join the epithelial cells (Fig. 5), and occasional hemidesmosomes link the peritoneum to the underlying basal lamina.

The outer connective tissue layer, delimited on either side by basal laminae, contains striated collagen fibrils and unstriated fibrils embedded in an interfibrillar matrix (Figs. 5, 7). Amoebocytes and cell processes are occasionally seen in the connective tissue (Fig. 7).

The genital coelom varies in width and often has reduced dimensions due to the presence of large vitellogenic oocytes in the germinal layer. Apart from a few coelomocytes and cell debris (Fig. 5), the coelom appears devoid of contents. In life, however, the coelom is presumably a fluid-filled space. A squamous epithelium lines the coelom and the inner coelomic epithelium includes circular and longitudinal myoepithelial cells (Fig. 6). The circular musculature of the inner sac is the major muscle layer of the ovary. Cytoplasmic contents of the coelomic epithelial cells include an elongate nucleus, ribosomes, vesicles, microtubules, rough endoplasmic reticulum (RER), and Golgi complexes (Figs. 5, 7). It was noted that a few of the cells were ciliated. Nerves are present in the outer and inner coelomic epithelia (Figs. 5, 7, 35), and large granulated processes are a prominent feature of the inner epithelium (Fig. 5). The epithelial cells are joined to the underlying basal lamina by hemidesmosomes.

The inner connective tissue layer is delimited by basal laminae that have a tortuous outline (Figs. 5–7), although the inner basal lamina is straight when juxtaposed with vitellogenic oocytes (Figs. 6, 35, 37). As for the outer sac, the inner connective tissue contains collagen fibrils and unstriated fibrils (Fig. 5). The genital haemal sinus is also a component of the inner connective tissue layer, but is only evident next to vitellogenic oocytes. Haemal fluid accumulates around the vitellogenic oocytes (Fig. 4, 6), but is absent from portions of the connective tissue next to previtellogenic and residual oocytes (Figs. 5, 7). The fluid stains pale purple with Azur II/methylene blue (Figs. 4, 8) and has a flocculent, granular appearance (Figs. 6, 38). Fibrillar elements of the connective tissue are also embedded in the haemal fluid (Figs. 6, 38). Amoebocytes are occasionally encountered in the inner connective tissue (Fig. 5).

The germinal epithelium consists of germinal and so-

Figure 8. LM cross section near the base of the ovary showing a group of early germinal cells (EG), including dividing oogonia (arrows). H, haemal fluid, LO, late vitellogenic oocyte, S, somatic cell, Y, yolk body. Scale = 22 μm.

Figure 9. Mitotic profile (arrow) between midvitellogenic oocytes (MO). Scale = 40 μm.

Figure 10. Early germinal cells (EG) lying alongside and *not* in an evagination of the inner connective tissue layer (IC). G, Golgi complex, L, lipid droplet, N, nucleus, S, somatic cell. Arrow, basal process. Scale = 4.0 μm.

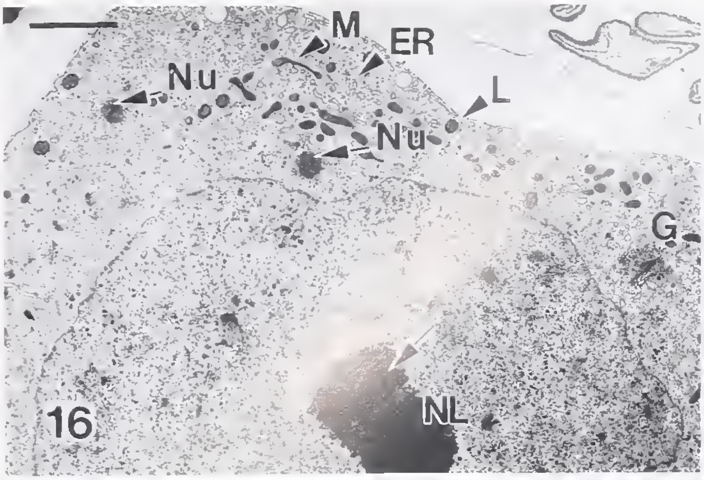
Figure 11. The somatic cells (S) partially surround the early germinal cells (EG) and have an abundance of vesicles (V) in their cytoplasm. G, Golgi complex, M, mitochondrion, N, nucleus. Scale = 4.0 μm.

Figure 12. Small extrusions of the somatic cell (arrows) are closely applied to the oocyte (O). L, lipid droplet, M, mitochondrion, N, nucleus. Scale = 1.0 μm.

Figure 13. A somatic cell not associated with an oocyte. C, cilium, G, Golgi complex, M, mitochondrion, Mt, microtubule, V, vesicles. Scale = 1.5 μm.

Figure 14. Previtellogenic oocyte in a depression of the inner connective tissue layer (IC). ER, endoplasmic reticulum, L, lipid droplet, LO, late vitellogenic oocyte, M, mitochondria, N, nucleus, Nu, nuage, Y, yolk body. Scale = 5.0 μm.

Figure 15. Nucleocytoplasmic exchange is indicated by the basophilic material (arrows) emerging from the nuclear pores (NP). N, nucleus. Scale = 1.0 μm.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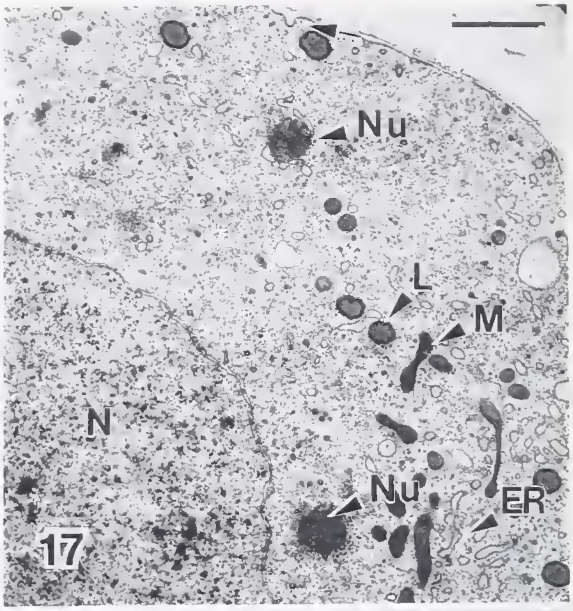
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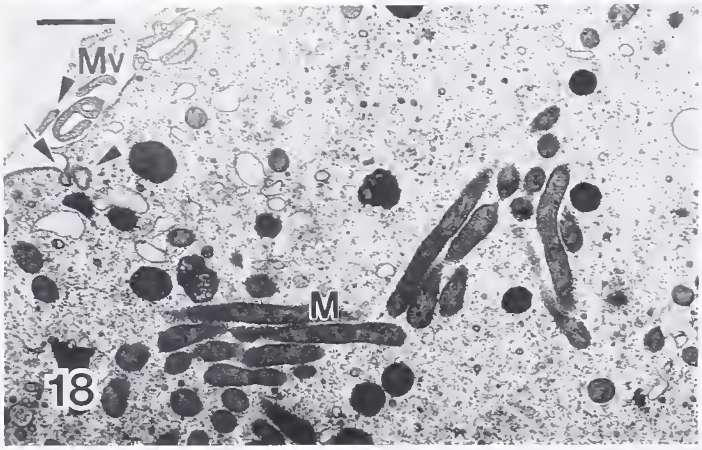
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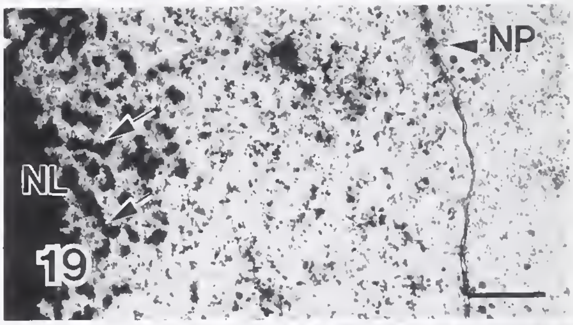
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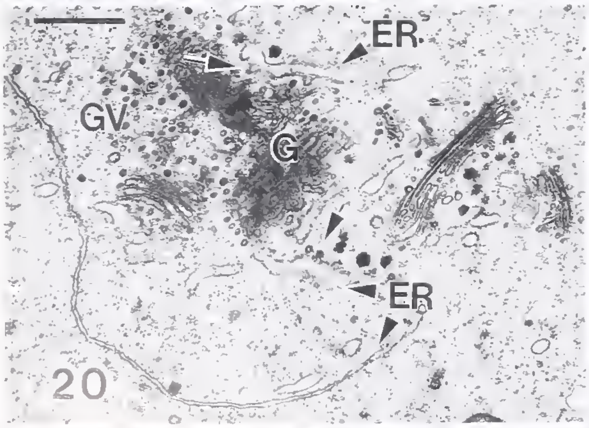
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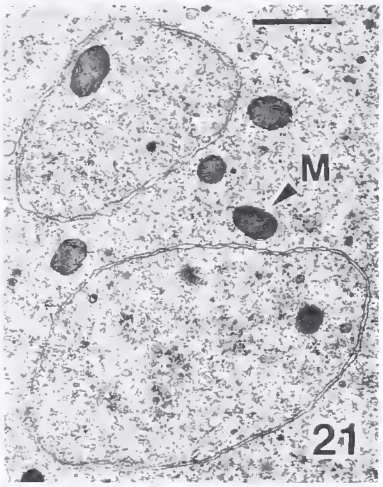
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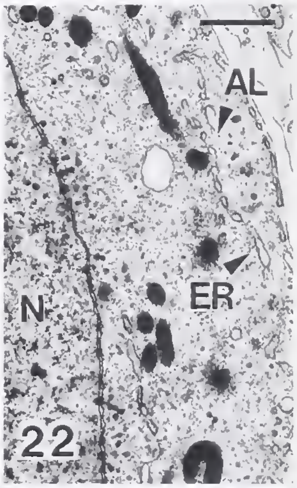
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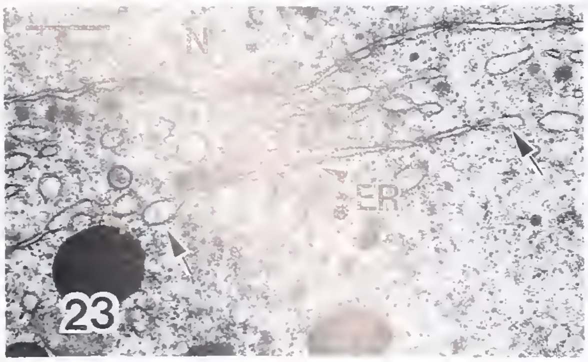
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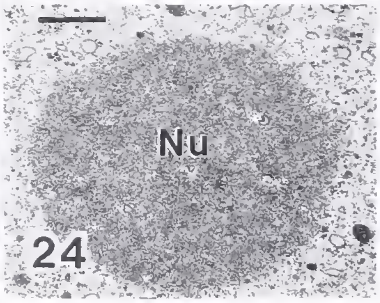
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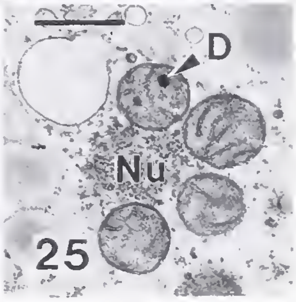
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matic cells that follow the contour of the inner basal lamina (Figs. 5, 10). The ovary has a poorly organized appearance because the oogenic stages are not spatially segregated (Fig. 4). In addition, the attachment of the developing oocytes with the germinal epithelium becomes reduced to a short stalk (Fig. 26).

Groups of small germinal cells (15–25 μm in length) are present at the base of the ovary near its attachment to the genital plate (Figs. 4, 8). The small germinal cells include proliferating oogonia in various stages of mitosis and early primary oocytes (Fig. 8). Oogonial proliferation occurs elsewhere in the ovary and mitotic profiles were also seen in cells adjacent to vitellogenic oocytes (Fig. 9). The germinal cells with few or no nuclear pores may be oogonia (Fig. 10), based on the criteria used in other studies (Piatigorsky, 1975; Kanatani and Nagahama, 1983). Characteristic features of the small germinal cells include a large nucleus (8–15 μm diameter) and a small amount of cytoplasm (Figs. 10, 11). The nucleus contains one or two nucleoli and patches of clumped chromatin. Cytoplasmic organelles include numerous free ribosomes, groups of mitochondria, vesicles, Golgi complexes and occasional lipid droplets (Figs. 10–12).

The somatic cells (8–17 μm long) of the germinal epithelium are squamous, irregularly shaped, and ciliated (Figs. 10–13). They are distinguished from the germinal cells by their small, dark nucleus (6–11 μm length) with chromatin lining the nuclear envelope (Fig. 12). Somatic cells also contain an abundance of electron-lucent vesicles, mitochondria, a Golgi complex, microtubules, lipid droplets, and relict oocyte material (Figs. 10–13). Cytoplasmic extensions of the somatic cells interdigitate with each other and form an incomplete follicle around the germinal cells (Figs. 10, 11). Small extrusions from the somatic cells are closely applied to the oocyte surface, but junctional complexes were not observed (Fig. 12). The somatic cells do not appear to be involved in oocyte nutrition and their association with the oocytes terminates early in oogenesis.

Previtellogenic oocytes

Early previtellogenic oocytes (30–50 μm diameter) are attached to the basal lamina or to neighboring cells by a basal process (Figs. 5, 10). As the oocytes grow, they press against the ovary wall forming a depression in the inner connective tissue (Figs. 14, 45). Previtellogenic oocytes have a prominent germinal vesicle containing a single nucleolus and dispersed chromatin (Fig. 16). During the previtellogenic stage there is an increase in the size of the germinal vesicle and in the amount of cytoplasm. The nucleolus has two regions, an electron-dense core and a peripheral nucleolonema that is granular and emanates chromatin-like strands (Figs. 16, 19). The nuclear envelope is perforated by annulated pores that appear to be a site for nucleocytoplasmic exchange, indicated by the basophilic material emerging from the pores (Figs. 15, 19).

Early previtellogenic oocytes contain free ribosomes, mitochondria, lipid droplets, microtubules, Golgi complexes, and some ER (Figs. 16–18). Mitochondriogenesis occurs in the previtellogenic stage, with an abundance of pleomorphic mitochondria that are often concentrated in a 'mitochondrial cloud' (Fig. 18). Many of the mitochondria have an eccentrically placed dense granule (Fig. 25). Golgi complexes located in the perinuclear ooplasm (Fig. 16) increase in number and dominate this region of the oocyte (Fig. 20) and as oogenesis proceeds, the Golgi disperse towards the cortical ooplasm. Each Golgi complex consists of 7–9 cisternae and many of them have a single cisterna of ER positioned next to the forming face (Fig. 20). Golgi vesicles containing electron-dense material arise from the maturing face of the Golgi and the ER also gives rise to electron-dense vesicles (Fig. 20). Elon-

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Figures 16–26. Previtellogenic oocytes.

Figure 16. The nucleolus (NL) is bipartite with a peripheral nucleolonema (arrow). ER, endoplasmic reticulum, G, Golgi complex, L, lipid droplet, M, mitochondrion, Nu, nuage. Scale = 3.0 μm.

Figure 17. The nuage (Nu) is surrounded by endoplasmic reticulum (ER) or is independent. L, lipid droplet, M, mitochondrion, N, nucleus. Arrow, endocytotic profile. Scale = 2.0 μm.

Figure 18. A coated pit (arrow) and coated vesicle (arrowhead) in a previtellogenic oocyte. M, mitochondrial cloud, Mv, microvillus. Scale = 1.0 μm.

Figure 19. Portion of the nucleolus (NL) with peripheral strands (arrows). The nuclear envelope is partially perforated by pores (NP). Scale = 1.0 μm.

Figure 20. Perinuclear Golgi complexes (G) and associated endoplasmic reticulum (ER). The ER gives rise to transport vesicles (arrows). Note the long ER cisterna. GV, Golgi vesicles. Scale = 1.0 μm.

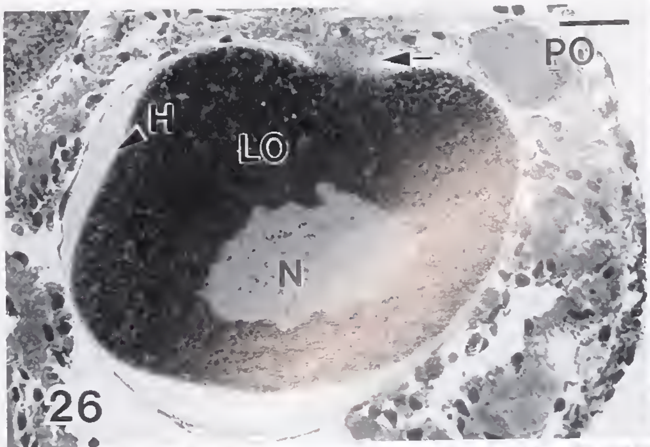
Figure 21. Circular endoplasmic reticulum, M, mitochondrion. Scale = 1.0 μm.

Figure 22. Single profile of annulate lamellae (AL) and associated endoplasmic reticulum (ER). N, germinal vesicle. Scale = 1.0 μm.

Figure 23. ER cisterna with dilated tips (arrows). N, nucleus. Scale = 0.5 μm.

Figure 24. The large nuage (Nu) seen in Fig. 14. Scale = 1.0 μm.

Figure 25. Small nuage (Nu) surrounded by mitochondria. D, dense granule. Scale = 1.0 μm.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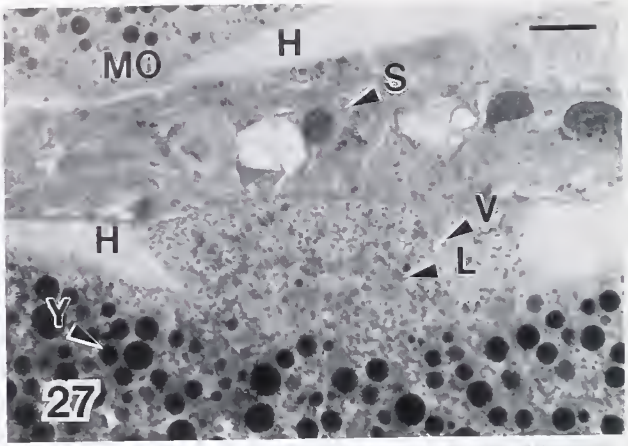
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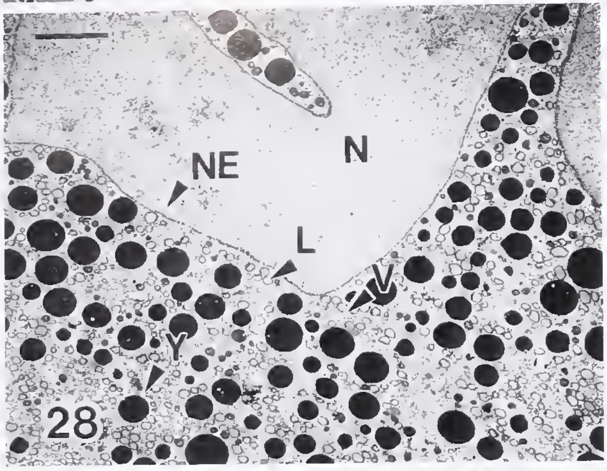
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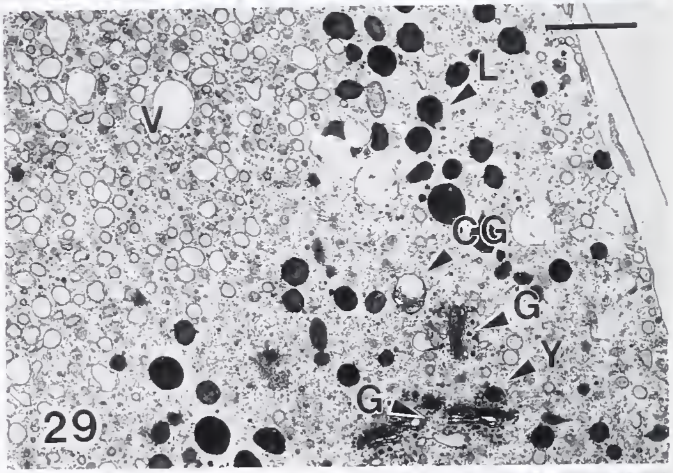
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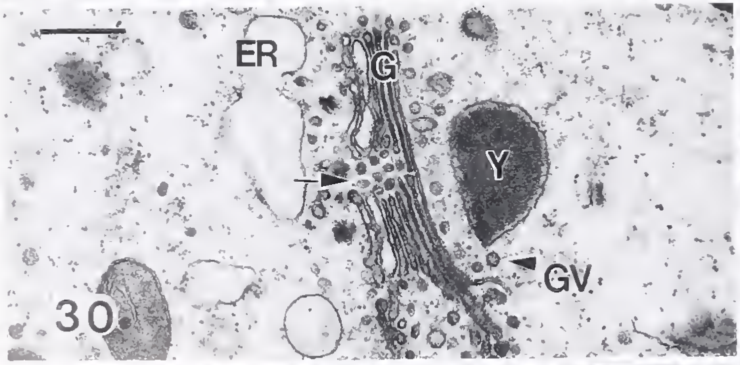
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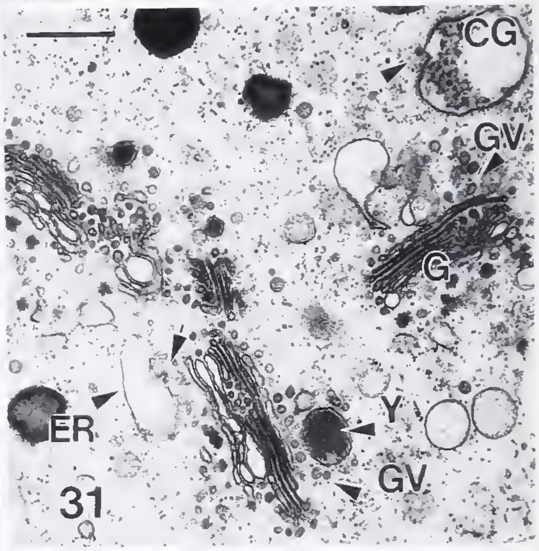
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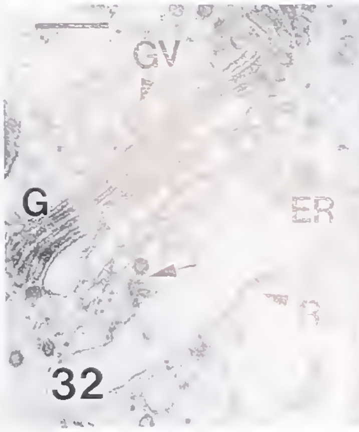
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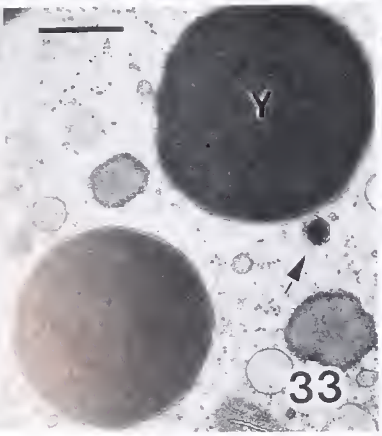
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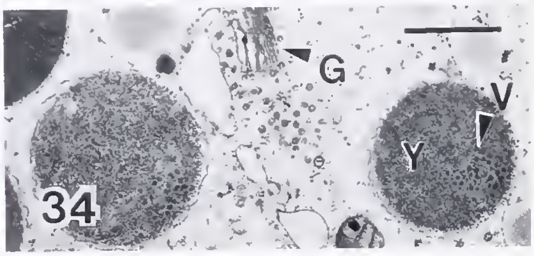
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gate and circular ER are scattered in the cytoplasm (Figs. 14, 20, 21). All the ER in the early oocytes appears to lack attached ribosomes (Figs. 20, 21). Dilated tips of the ER cisternae give rise to small vesicles (Fig. 23) and appear to form single profiles of annulate lamellae (Fig. 22). Evidence of ER vesiculation is present throughout previtellogenesis and membranous, electron-lucent vesicles are a prominent feature of late previtellogenic and early vitellogenic oocytes (Fig. 29). The function of these vesicles is not known.

Fibrogranular material that lacks a unit membrane, similar to the nuage seen in other oocytes (Eddy, 1975; Wourms, 1987), is common in previtellogenic oocytes (Figs. 14, 17). This material, possibly derived from nucleocytoplasmic transfer, is present as large (4–5 μm diameter, Figs. 14, 24) and small (0.3–1.0 μm diameter, Figs. 17, 25) fibrogranular bodies (FGBs). The small FGB's are often surrounded by mitochondria or ER cisternae (Figs. 17, 25). During oogenesis the FGB's disperse.

The number of lipid droplets (0.3–0.8 μm diameter) increases throughout oogenesis and they appear to arise *de novo* in the cytoplasm. Lipid droplets are not membrane-bound and are dispersed throughout the ooplasm (Fig. 28). They are not associated with other organelles and it is unknown how they originate.

Microvilli are elaborated in local patches along the oolemma of late previtellogenic oocytes and endocytosis is evident with coated pits (150–200 μm) present at the bases of the microvilli (Fig. 18). Endocytosis also occurs in early previtellogenic oocytes that lack microvilli (Fig. 17).

Vitellogenic oocytes

The onset of the vitellogenic period is marked by the presence of yolk bodies in the ooplasm, starting with oocytes approximately 100 μm in diameter (Figs. 4, 26). As the oocytes increase in size, they bulge outwards, causing the inner basal lamina to unfold and follow the contours of the oocyte (Figs. 35, 37, 45). They become increasingly independent of the germinal layer with each vitellogenic oocyte residing in a sac-like depression of the inner connective tissue and attached to the germinal epithelium by a neck-like stalk (Figs. 26, 27). The attachment region contains lipid droplets, electron-lucent vesicles, and is usually devoid of yolk bodies (Fig. 27). Continued oocyte growth causes attenuation of the inner basal lamina, whereas the outer basal lamina is thicker and plicated (Figs. 6, 35). The presence of an attachment stalk in advanced oocytes suggests that the oocytes retain their association with the germinal epithelium throughout vitellogenesis.

Haemal fluid accumulates in the connective tissue layer adjacent to vitellogenic oocytes (Figs. 4, 26), starting in early vitellogenesis. Although each oocyte is surrounded by haemal fluid, it remains separated from the fluid by the inner basal lamina (Figs. 35, 37). The association between the genital haemal sinus and the oocytes is particularly evident during late and mid-vitellogenesis when haemal fluid accumulates in depressions of the oocytes and on either side of the attachment region (Figs. 4, 26, 27). However, haemal fluid is not restricted to these areas. Serial sections of the ovary revealed that the haemal investment around each oocyte is not uniform

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Figures 26–34. Vitellogenic oocytes.

Figure 26. A late vitellogenic oocyte (LO) in a sac-like evagination of the inner connective tissue layer, surrounded by haemal fluid (H) and attached to the germinal layer by a short stalk (arrow). N, germinal vesicle, PO, previtellogenic oocyte. Scale = 50 μm.

Figure 27. Stalk connecting the oocyte to the germinal layer. H, haemal fluid, L, lipid droplet, MO, midvitellogenic oocyte, S, somatic cell, V, vesicle, Y, yolk body. Scale = 9.5 μm.

Figure 28. The nuclear envelope (NE) is convoluted and the germinal vesicle (N) appears amorphous. L, lipid droplet, V, vesicle, Y, yolk body. Scale = 5.0 μm.

Figure 29. Early vitellogenic oocyte with the cytoplasm dominated by electron-lucent vesicles (V). One Golgi complex (G) is beside a small yolk body (Y) and the other beside a cortical granule (CG). See Figure 31. L, lipid droplet. Scale = 3.0 μm.

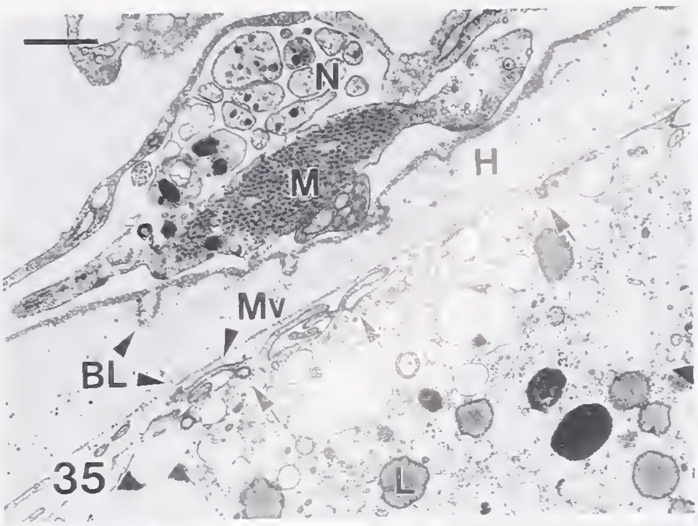
Figure 30. Yolk body (Y) formation in an early vitellogenic oocyte. Transport vesicles (arrow) derived from the endoplasmic reticulum (ER) appear to fuse with the forming face of the Golgi complex (G), which also gives rise to vesicles (GV). Scale = 0.5 μm.

Figure 31. Golgi (G) region seen in Fig. 29. ER-projections (arrow) give rise to vesicles and Golgi vesicles (GV) appear to fuse with the yolk body (Y) and with (arrowhead) the cortical granule (CG). Scale = 0.75 μm.

Figure 32. Portion of the endoplasmic reticulum (ER) with attached ribosomes (R) in a midvitellogenic oocyte. ER-projections (arrow) form transport vesicles that appear to fuse with the forming face of the Golgi (G). GV, Golgi vesicles. Scale = 0.3 μm.

Figure 33. Dense yolk body (Y) and attached vesicle (arrow) in a midvitellogenic oocyte. Scale = 0.6 μm.

Figure 34. Granular yolk body (Y) with vesicular inclusions (V) in a midvitellogenic oocyte. G, Golgi complex. Scale = 1.0 μm.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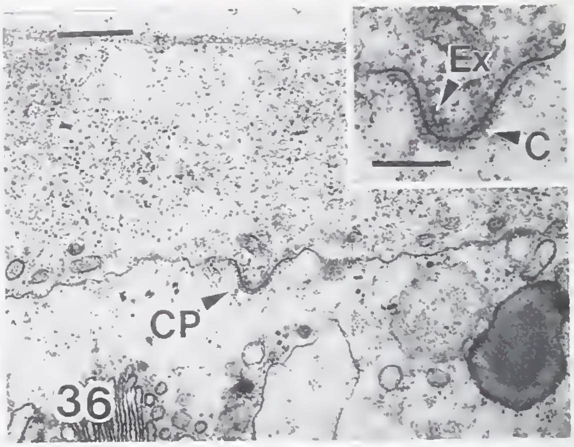
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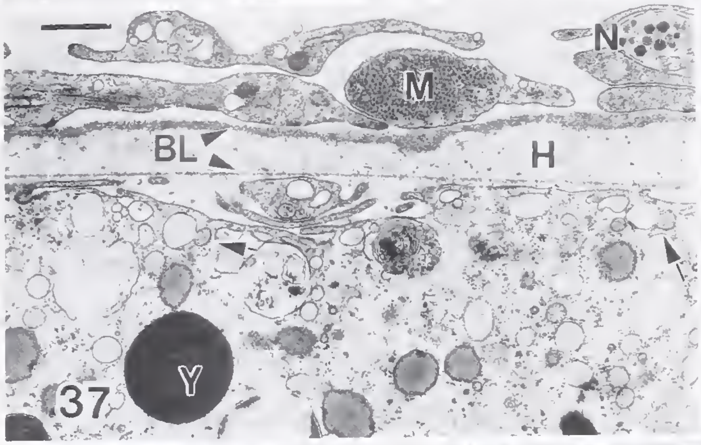
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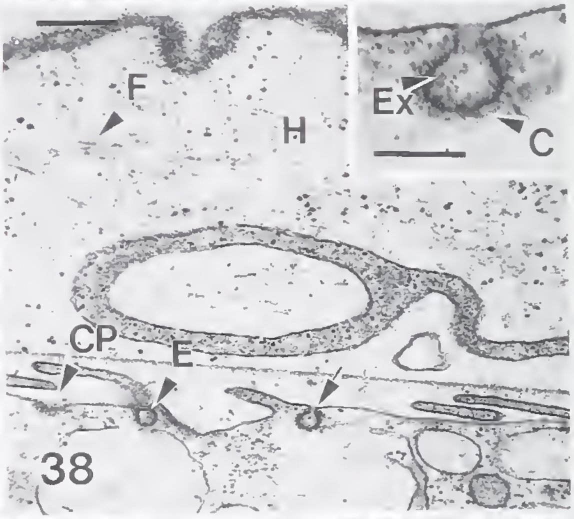
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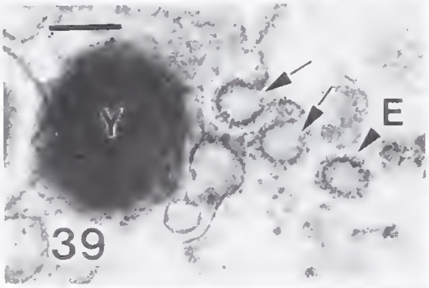
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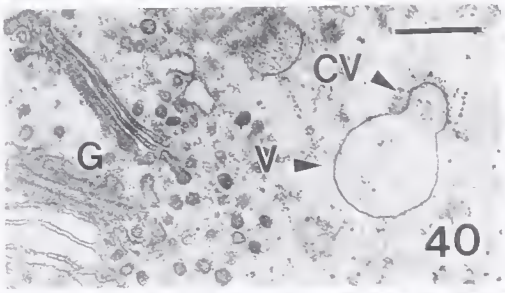
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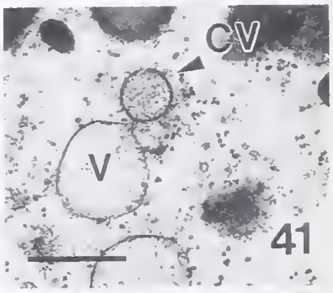
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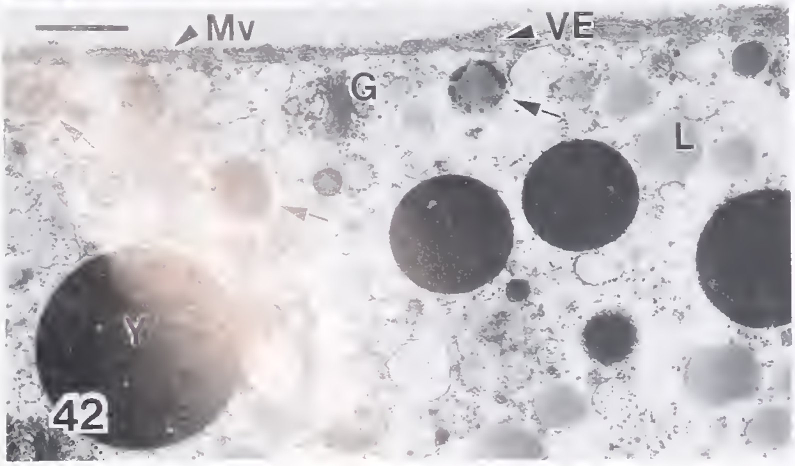
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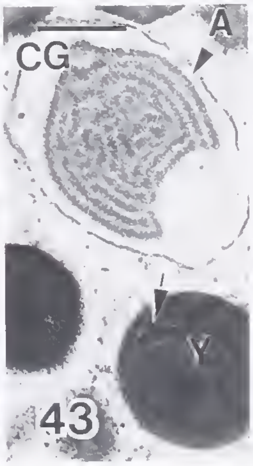
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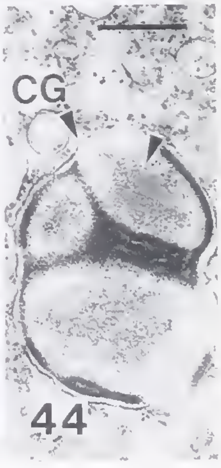
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44

and that the fluid does not appear to exhibit an affinity for any particular region of the oocytes.

The space between the oolemma and the inner basal lamina appears devoid of contents in oocytes fixed with seawater buffered glutaraldehyde (Figs. 35, 37). However, with the cacodylate method flocculent material reminiscent of haemal fluid is occasionally present in this space (Fig. 36).

During vitellogenesis, the volumes of the ooplasm and the germinal vesicle continue to increase (Figs. 26, 28). Early vitellogenic oocytes contain small yolk bodies (0.6–1.6 μm diameter), lipid droplets, mitochondria, cortical granules, electron-lucent vesicles, nuage, Golgi complexes, ER, and free ribosomes; the nuclear envelope is undulated (Figs. 26, 28). Portions of the ooplasm dominated by the membranous vesicles encountered in the previtellogenic stage are markedly devoid of yolk bodies (Fig. 29). These vesicles subsequently disperse through the ooplasm (Fig. 28). In midvitellogenic oocytes, Golgi complexes abound in the cortical ooplasm (Fig. 29). As in previtellogenic oocytes, most of the ER appears to be smooth (Figs. 30, 31), however patches of ER with attached ribosome-like particles were observed in cisternae associated with a Golgi complex (Fig. 32). These patches of rough ER on otherwise smooth cisternae are rare and only evident in vitellogenic oocytes. In vitellogenic oocytes, ER is present only as short cisternae adjacent to a Golgi complex.

In early vitellogenic oocytes, yolk formation occurs in the perinuclear ooplasm, whereas in late vitellogenic oocytes yolk formation is largely cortical. Newly formed yolk bodies (0.14–0.80 μm length) are positioned near a Golgi complex (Figs. 30, 31). They have an electron-dense or granular core surrounded by an electron-lucent, halo-like cortex. It appears that the Golgi complex and the ER are involved in the elaboration of the yolk bodies. The process starts with small expansions of the ER cisternae that project towards the Golgi complex (Figs. 31, 32). These projections often have an external coat and their detachment gives rise to transport vesicles that are coated. A flocculent material is contained within the vesicles and they appear to fuse with the forming face of the Golgi (Fig. 30). Vesicles containing material of variable electron density are released from the maturing face of the Golgi and fuse to form yolk bodies (Figs. 30–32). During the vitellogenic stage, the yolk bodies continue to grow through successive incorporation of material contained within Golgi vesicles (Figs. 30, 31, 33). Midvitellogenic oocytes contain yolk bodies (0.7–2.5 μm diameter) of variable electron density. Growing yolk bodies often have a light halo and may contain membranous strands (Fig. 43). Some yolk bodies are light and granular with vesicular inclusions (Fig. 34), whereas others are dark and homogeneous (Fig. 33). As vitellogenesis proceeds, the yolk material condenses and the cytoplasm of advanced oocytes (300–400 μm diameter) is dominated by yolk bodies that are uniformly electron-dense and 2–5 μm in diameter (Fig. 28).

The oolemma changes throughout vitellogenesis with the continued elaboration of microvilli, which have a scattered distribution around the oocyte. Endocytotic activity is present throughout vitellogenesis and is particularly conspicuous at the bases of the microvilli with up to three coated pits per μm of oolemma (Figs. 35–39). The cytoplasmic surface of the pits has a fuzzy or spiked appearance and flocculent material adheres to the extracellular surface (Figs. 36, 38, 39). The pit and adherent material appear to invaginate to form a cup-like depres-

Figures 35–44. Late vitellogenic oöcytes.

Figure 35. Endocytotic activity along the oolemma (arrows). BL, basal lamina, H, haemal fluid, L, lipid droplet, M, muscle, Mv, microvillus, N, nerve. Scale = 1.0 μm.

Figure 36. The cup-shaped coated pit (CP) has a spiked cytoplasmic surface (C, insert) and flocculent material which surrounds the oocyte adheres to the extracellular surface of the coated pit (Ex, insert). The fixative was buffered with cacodylate. Scale = 1.0 μm. Insert scale = 0.2 μm.

Figure 37. Endosomes appear to fuse with smooth vesicles in the cortical ooplasm (arrows). BL, basal lamina, H, haemal fluid, M, muscle, N, nerve, Y, yolk body. Scale = 1.0 μm.

Figure 38. Formation of an endosome. A coated pit (CP) is invaginated to form an omega-shaped pit (arrow and insert) and internalized to form a coated vesicle. The cytoplasmic side (C) of the pit has a fuzzy appearance and flocculent material on the extracellular surface (Ex) is being internalized (insert). The fixative was buffered with seawater. F, collagen fibril, H, haemal fluid. Scale = 0.5 μm. Insert scale = 0.15 μm.

Figure 39. Three endocytotic profiles; two omega figures (arrows) and an endosome (E), Y, yolk droplet. Scale = 0.2 μm.

Figures 40–41. Coated (CV) and smooth vesicles (V) fusing in the Golgi (G) area. Scale = 0.5 μm.

Figure 42. Peripheral ooplasm of an oocyte fixed with the cacodylate method. The cortical granules (arrows) have contrasting dark and light areas. G, Golgi complex, L, lipid droplet, Mv, microvillus, VE, vitelline envelope, Y, yolk body. Scale = 1.5 μm.

Figures 43–44. Cortical granules (CG) of oocytes fixed with the seawater method with annular (A) and amorphous (arrowhead) contents. Arrow, membranous strand in the yolk (Y) body. Scale 0.5 μm.

sion or an omega figure (Figs. 36, 38, 39) and are then internalized, forming a coated vesicle (endosome). Newly formed endosomes are common near the oolemma (Figs. 37, 39). In Figure 37, the flocculent material surrounding the oocyte is present in the pit and appears to be incorporated by the oocyte. The endosomes appear to lose their cytoplasmic coat soon after internalization and are indistinguishable from other smooth vesicles that abound in the cortical ooplasm. There is evidence that the coated vesicles fuse with smooth vesicles near the oolemma (Fig. 37). Coated vesicles fusing with smooth vesicles are also present in the Golgi region (Figs. 40, 41), but it is not known if they are derived from coated pits. The fate of the material internalized through endocytosis could not be followed.

Cortical granule formation is initiated in the early vitellogenic stage. In these oocytes, cortical granules are found in the endoplasm and in the cortical ooplasm. Late vitellogenic oocytes have abundant cortical granules (1.4–1.6 μm diameter) in the peripheral ooplasm (Fig. 42). The cortical granules are membrane-bound, irregular in shape, and contain material with differing staining densities (Figs. 42–44). Their appearance is influenced by the method of fixation. In material preserved with cacodylate-buffered glutaraldehyde, the granules have contrasting dark and light regions and contain a flocculent material (Fig. 42). However, in oocytes fixed with the seawater method, the cortical granules are less electron-dense and the contents have an annular or flocculent appearance (Figs. 43, 44). Some of the cortical granule material may originate from ER-Golgi activity. Golgi-derived vesicles appear to coalesce with the cortical granules adjacent to the oolemma (Fig. 31).

A vitelline envelope is evident in advanced oocytes fixed with the cacodylate method (Fig. 42). The envelope consists of dark fibrous material closely applied to the oolemma, ranging in diameter from 60 to 300 nm. Additional extracellular coats, including a jelly layer, were not observed but may be deposited in the post-vitellogenic stage in the ovarian lumen prior to spawning.

Discussion

The ovary wall

The ovary of *Ophiolepis paucispina* is morphologically similar to that of other ophiuroids and to the ovaries of asteroids and echinoids that also have a two-sac structure (Davis, 1971; Walker, 1979, 1982). Because the ovary is continually expanded with advanced oocytes, the epithelial layers of the ovary have an attenuated profile. Flagellated-collar cells present in the peritoneum of asteroid ovaries (Walker, 1979) are not present in *O. paucispina*, and, in general, there appears to be a paucity of ciliated cells in the ovarian peritoneum. Suspensor cells, similar to those seen in the ovary, also connect with the testes of *O. paucispina* and with the testes of *Amphipholis squamata* (Buckland-Nicks *et al.*, 1984; pers. obs.). The epithelial layers of the ovary of *O. paucispina* contain myoepithelial cells, a characteristic of echinoderm coelomic epithelia (Rieger and Lombardi, 1987). By contrast, Davis (1971) did not report the presence of musculature in the ovarian coelomic epithelia of *Ophioderma panamense* Lütken and *Ophiopteris papillosa* (Lyman). Contraction of the ovarian musculature presumably assists spawning, which may occur through rupture of the ovary wall, as suggested for other ophiuroids that lack an oviduct (Davis, 1971).

The ovary of *Ophiolepis paucispina* is well innervated by neurons that run beside the myoepithelial cells. These neurons contain vesicles characteristic of neurons associated with echinoderm muscles (Cobb, 1987), and may innervate the ovarian musculature. Large granulated processes are common in the inner coelomic epithelium and are similar to the neurosecretory-like cells common in echinoderm epithelia (Cobb, 1987). The function of these processes is not known, but their structure and position indicates that they may serve an endocrine function in oocyte development, as suggested for asteroid, crinoid, and holothuroid species (Holland, 1971; Ferrand, 1983; Smiley and Cloney, 1985). The connective tissue layers of the ovary of *O. paucispina* are primarily supportive and the inner connective tissue contains the genital haemal sinus which surrounds vitellogenic oocytes.

Germinal layer and the role of somatic cells

Because oocytes at all stages of development are interspersed in the ovary of *Ophiolepis paucispina*, a distinct germinal layer is not apparent. Oogonial proliferation occurs near the base of the ovary, similar to *Ophioderma brevispinum* (Say) and *Ophiura albida* Forbes (Tyler, 1976; Hendler and Tyler, 1986), but also takes place elsewhere in the ovary, suggesting that there may not be a discrete proliferative region. For *O. paucispina* it is not known where the primordial germ cells originate. In *Gorgonocephalus eucnemis* (Müller and Troschel), the primordial germ cells enter the ovary through haemal channels and oocyte proliferation occurs in the genital haemal sinus (Patent, 1976). Similarly, the primordial germ cells of a holothuroid species are located in the connective tissue at the base of the ovary (Smiley, 1988).

Oogenesis in echinoderms may be solitary or follicular (Walker, 1982), and, although the small germinal cells and early oocytes of *Ophiolepis paucispina* are covered by epithelial cells, oogenesis is solitary. The somatic cells appear to provide structural support for the early oocytes and may contribute to the microenvironment required for early development. In the vitellogenic stage, the somatic cells along the attachment site also appear to play a supportive role. The oocytes of *Ophioderma brevispinum* are also surrounded by follicle cells at an early stage and their association with the germinal layer during vitellogenesis appears to be limited to a short stalk (Hendler and Tyler, 1986). In contrast, the oocytes of *Gorgonocephalus eucnemis* are surrounded by an incomplete follicle throughout their development and attached nurse cells appear to be involved with oocyte nutrition (Patent, 1968). Crinoid oogenesis is also solitary and is strikingly similar to that seen in *O. paucispina*. In several crinoid species the oocytes arise among follicle cells, but oogenesis proceeds with each oocyte in an evagination of the haemal sinus and attached to the germinal layer by a short stalk (Holland *et al.*, 1975; Mladenov, 1986). Accessory cells free in the genital haemal sinus may be involved in oocyte nutrition (Holland and Kubota, 1975).

For the other echinoderm classes, oogenesis appears to be follicular. In asteroids the oocytes are held within a follicle throughout their development (Schroeder *et al.*, 1979; Kanatani and Nagahama, 1983; Beijnink *et al.*, 1984c) and the follicle cells are a potential source of nutrients for oogenesis (Chia, 1968; Beijnink *et al.*, 1984a). Echinoid oocytes are surrounded by accessory cells that provide nutrients for oocyte growth (Walker, 1982; Kanatani and Nagahama, 1983). In holothuroids where oogenesis of one species has been examined, the oocytes develop within a follicle (Smiley and Cloney, 1985; Smiley, 1988).

Oogenesis

Many of the cellular events that occur in the early stages of oogenesis in *Ophiolepis paucispina* are characteristic of the previtellogenic oocytes of echinoderms and other animal groups (Norrevang, 1968; Anderson, 1974; Piatigorsky, 1975; Kanatani and Naganama, 1983; Eckelbarger, 1984; Wourms, 1987). Comparative information on oocyte ultrastructure is available for several ophiuroids (Kessel, 1968; Patent, 1968; Davis, 1971; Bell, 1974; Tyler, 1976). During previtellogenesis of *O. paucispina, Ophioderma panamense,* and *Ophiura albida,* the nucleolus appears to 'spin-out' to form a nucleolonema (Kessel, 1968; Tyler, 1976), a structure thought to be involved in nucleocytoplasmic transport of ribosomes (Kessell, 1968). Nuage deposits similar to those seen in *O. paucispina* are present in the oocytes of several echinoderms, and may be germ cell determinants useful for the identification of germ line cells (Eddy, 1975; Wourms, 1987; Smiley, 1988).

Single profiles of annulate lamellae, similar to those seen in *Ophiolepis paucispina* oocytes, are present in *Acrocnida brachiata* (Montagu) oocytes (Bell, 1974), but differ from the parallel stacks of lamellae seen in the oocytes of *Ophioderma panamense* and those of several holothuroid, crinoid, and echinoid species (Kessel, 1966, 1968; Holland, 1971; Piatigorsky, 1975). Membranous vesicles are a prominent feature of the previtellogenic and early vitellogenic oocytes of *O. paucispina* and appear to be at least partially derived from ER vesiculation. Some of the vesicles may be empty pigment granules, as suggested for the oocytes of an echinoid (Anderson, 1974). The oocytes of *O. paucispina* have a conspicuous pink color in life, but pigment bodies are not evident upon ultrastructural examination. A similar extensive system of vesicles in the oocytes of *O. panamense* gives rise to stacks of annulate lamellae (Kessel, 1968).

Compared with the oocytes of several echinoid, holothuroid, and crinoid species, the oocytes of *Ophiolepis paucispina* have a relatively small amount of ER, and, moreover, the ER in these other oocytes is clearly rough surfaced (Kessel, 1966; Verhey and Moyer, 1967; Bal *et al.*, 1968; Holland, 1971; Aisenshtadt and Vassetzky, 1986). Kessel (1968) notes that the number of ribosomes associated with ER is low in *Ophioderma panamense* oocytes, and describes small patches of rough ER, similar to those seen in this investigation. The presence of small patches of rough ER suggests that the association between the ribosomes and ER cisternae may be transient (Kessel, 1968). Alternatively, the paucity of rough ER may be an artifact resulting from fixation-induced detachment of ribosomes (Eckelbarger, pers. comm.).

Proteid yolk deposition in *Ophiolepis paucispina* is first evident in the Golgi region and appears to result from a sequence of ER-Golgi activity similar to that described for the oocytes of *Ophioderma panamense* and for those of several echinoderms (Kessel, 1966, 1968; Chatlynne, 1972; Piatiagorsky, 1975; Ferrand, 1984; Aisenshtadt and Vassetzky, 1986). In vitellogenic oocytes, the differential distribution of ER in the Golgi region appears to be a functional relationship for yolk deposition. The whorls of ER seen in the oocytes of other ophiuroids (Kessel, 1968; Bell, 1974, Tyler, 1976), thought to be involved in lipid deposition, were not seen in the oocytes of *O. paucispina.*

The cortical granules of *Ophiolepis paucispina* are similar to those seen in several ophiuroids (Kessel, 1968; Tyler, 1976; Holland, 1979; Yamashita, 1984), and appear to arise through the fusion of Golgi vesicles, as reported for *Ophioderma panamense* (Kessel, 1968) and two echinoid species (Anderson, 1974; Geary, 1978). Cortical granule contents are presumably exocytosed after fertilization, as occurs in *Ophiopholis aculeata* (O. F. Müller) and *Amphipholis kochii* Lütken (Holland, 1979; Yamashita, 1984). The influence of different fixation methods on the appearance of cortical granules is also

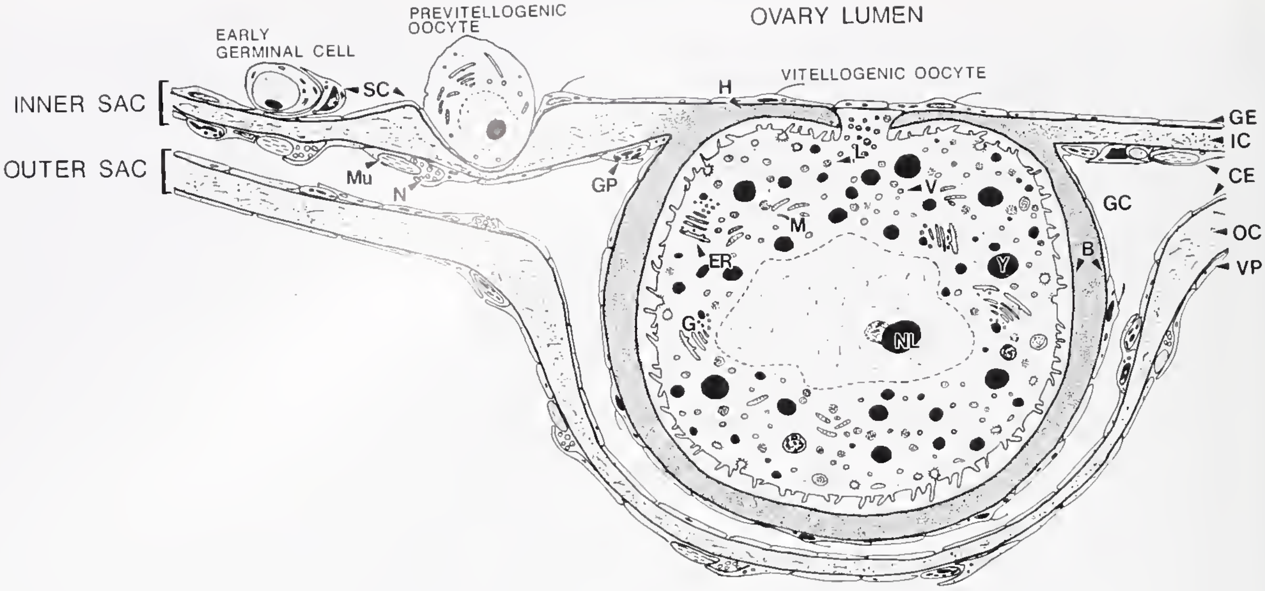

Figure 45. Diagrammatic illustration of a portion of the ovary of *Ophiolepis paucispina* summarizing oogenesis and the formation of the sac-like evagination in the inner connective tissue layer (IC). B, inner and outer basal laminae, ER, endoplasmic reticulum, G, Golgi complex, GC, genital coelom, GE, germinal epithelium, GP, granulated process, H, haemal fluid, L, lipid, M, mitochondria, Mu, muscle, N, nerve, OC, outer connective tissue, SC, somatic cell, VP, visceral peritoneum, Y, yolk body.

noted for the oocytes of several echinoid species (Takashima, 1968). In comparison with *O. aculeata* and *A. kochii* (Holland, 1979; Yamashita, 1984), the vitelline envelope of *O. paucispina* oocytes is thicker. This difference may be influenced by the method of fixation, as an extracellular coat was only preserved with the cacodylate method.

At the termination of oogenesis, the oocytes may ovulate into the ovarian lumen by squeezing through their apical aperture, as described for crinoid oocytes (Holland *et al.*, 1975; Mladenov, 1986).

Haemal sinus and oocyte nutrition

The close contact between the genital haemal sinus and the germinal layer is a characteristic feature of echinoderms (Davis, 1971; Walker, 1982). In *Ophiolepis paucispina* the oocytes bulge outwards as they grow and reside in an individual sac-like depression of the connective tissue layer throughout vitellogenesis (Fig. 45). Subsequently, haemal fluid accumulates around the oocytes. It is unclear whether the oocytes of other ophiuroids also sink into the connective tissue layer. The oocytes of *Gorgonocephalus eucnemis* are bathed in haemal fluid (Patent, 1968), but the details of the relationship between the oocytes and the haemal fluid were not described. In an ultrastructural study of the ovary wall, Davis (1971) reports that the oocytes of *Ophioderma panamense* are surrounded by haemal fluid, whereas those of *Ophiopteris papillosa* and *Ophiothrix spiculata* Le Conte are not. From the information provided (Davis, 1971), the significance of this difference cannot be assessed because oocytes at different stages of oogenesis may have been compared. From the results with *O. paucispina* oocytes, an increase in the volume of the genital haemal fluid would be expected to coincide with the vitellogenic phase. These differences in the relationship between ophiuroid oocytes and the genital haemal sinus warrant further examination through comparative ultrastructural investigations of ophiuroids at all stages of oogenesis.

The relationship between the genital haemal sinus and the developing oocytes of *Ophiolepis paucispina* is similar to that described for several crinoid species (Davis, 1971; Holland, 1971; Holland *et al.*, 1975). Crinoid oocytes also develop in a sac-like evagination and are separated from the surrounding haemal fluid by a basal lamina (Davis, 1971; Holland, 1971; Holland *et al.*, 1975). By contrast, asteroid oocytes are not surrounded by haemal fluid, but develop a close association with the haemal sinus along their basal stalk (Beijnink *et al.*, 1984c). In addition, the volume of the genital haemal sinus of asteroids increases, coincident with the oogenic cycle

(Chia, 1968; Walker, 1974). Holothuroid and echinoid oocytes are also juxtaposed with the genital haemal sinus along their basal surface (Davis, 1971; Smiley and Cloney, 1985). Moreover, echinoid oogenesis fails if the haemal connections of the ovary are severed (Okada, 1979; Pearse, 1982).

The dynamic changes in the genital haemal sinus of *Ophiolepis paucispina,* coincident with vitellogenesis, indicate that the haemal fluid may be a source of nutrients for oogenesis, as appears to be the case for several asteroid species (Walker, 1982). Furthermore, the prevalence of coated pits and vesicles along the oolemma indicates endocytotic uptake of exogenous material by the oocytes. The spike-like cytoplasmic coat of the pits is similar to that of pits involved in receptor-mediated endocytosis (Giorgi, 1980; Wild, 1980), and, based on the ultrastructural evidence of this study, it is suggested that yolk precursors may be selectively incorporated by the coated pits of *O. paucispina* oocytes. The haemal sinus appears to be the proximate source of these precursors which need only to pass through the basal lamina for access to the oocyte surface. Evidence for the presence of vitellogenic material in the genital haemal fluid of an asteroid species is reported in a recent immunocytochemical investigation (Beijnink *et al.,* 1984a). The presence of flocculent material around oocytes preserved with the cacodylate method suggests that haemal components may pass through the basal lamina. Furthermore, the material appears to be incorporated by the oocytes. It is not known if the absence of this material in oocytes fixed by the seawater method is due to extraction by the fixative.

As discussed in Byrne (1988), the nutritive material stored in the genital haemal sinus of *Ophiolepis paucispina* is presumably of somatic origin and is probably delivered to the ovary through the coelom. Coelomic translocation of nutrients and the absorptive properties of haemal fluid are demonstrated in several autoradiographic studies of asteroids where labelled amino acids were selectively concentrated by the haemal system (Ferguson, 1970, 1982, 1984; Beijnink and Voogt, 1984; Voogt *et al.,* 1985). Nutrient concentrations in echinoderm coelomic fluid is low (Ferguson, 1982; Beijnink *et al.,* 1984b) and it appears that the haemal sinus sequesters nutrients from coelomic circulation, and stores them, against a concentration gradient. Elevated concentrations of nutrients in the haemal sinus would also create a gradient favoring uptake of material by the oocytes. The apparent affinity of genital haemal fluid for nutrients and its differential accumulation around the vitellogenic oocytes of *O. paucispina* appear to be specialized features for a nutritive role in gametogenesis. The mechanisms underlying the dynamic properties of the haemal fluid, an extracellular material, remain to be elucidated.

Coated pits are short-lived (Dautry-Varset and Lodish, 1984), and the prevalence of endocytotic profiles in *Ophiolepis paucispina* oocytes suggests that incorporation of exogenous material is intense throughout vitellogenesis. Because the endosomes lose their extracellular coat soon after internalization and appear to fuse with pre-existing vesicles, the fate of the endocytosed material could not be followed. The material may be transferred directly to yolk bodies along with material synthesized by the oocytes or may be processed by the ER-Golgi complex. Endocytosis is reported for several echinoderm oocytes with coated pits typically located at the bases of microvilli (Takashima and Takashima, 1966; Bal, 1970; Holland, 1971; Ferrand, 1984; Smiley, 1988). By contrast, the absence of coated pits is noted for the oocytes of an ophiuroid (Bell, 1974) and several echinoid and asteroid species (Verhey and Moyer, 1967; Geary, 1978; Beijnink *et al.,* 1984c; Aisenshtadt and Vassetzky, 1986). However, the apparent absence of pits in these oocytes may be due to a low intensity of endocytotic uptake, which would limit the chances of finding coated pits. This is suggested for the asteroid *Asterias rubens* Linné, where the oocytes are known to incorporate exogenous material, but ultrastructural evidence of coated pits has not been found (Beijnink *et al.,* 1984c).

The foregoing speculations on the presence of vitellogenin in the genital haemal fluid and its pathway to the ovary of *Ophiolepis paucispina* are based on ultrastructural information alone and require verification by following the fate of labelled vitellogenin in this species with tracer techniques as in studies of insect and polychaete vitellogenesis (Giorgi, 1980; Fischer and Dhainaut, 1985; Baert and Slomianny, 1987). The prevalence of coated pits in the oocytes of *O. paucispina,* indicates that this ophiuroid may be a good subject for such an autoradiographic study.

In the terminology used to categorize vitellogenesis (Anderson, 1974; Eckelbarger, 1984; Wourms, 1987), yolk formation in *Ophiolepis paucispina* appears to be at least partially heterosynthetic with exogenous origin of the yolk precursors. However, the site of vitellogenin synthesis has not been determined. Free ribosomes and ribosomes associated with ER in the ooplasm may participate in the elaboration of yolk proteins (autosynthesis). Biochemical investigations of vitellogenin synthesis in echinoids demonstrate that yolk precursors are synthesized in the intestine, coelom, and gonad (Harrington and Ozaki, 1986; Ozaki *et al.,* 1986; Shyu *et al.,* 1986). Moreover, Harrington and Ozaki (1986) have shown that coelomocytes are a major source of vitellogenin in *Dendraster excentricus.* The accumulation of vitellogenin in the echinoid coelom provides further evidence for the coelomic transport of echinoderm yolk precursors (Harrington and Ozaki, 1986; Shyu *et al.,* 1986).

In summary, this investigation provides evidence for endocytotic incorporation of nutrients by the oocytes of *Ophiolepis paucispina* and adds to the accumulating evidence that the echinoderm genital haemal sinus is an intragonadal nutrient store (Walker, 1982; Ferguson, 1982, 1984; Kanatani and Nagahama, 1983; Voogt *et al.*, 1985). This, taken together with the biochemical evidence for the somatic and ovarian synthesis of vitellogenin (Harrington and Ozaki, 1986, Ozaki *et al.*, 1986; Shyu *et al.*, 1976) indicates that entirely autosynthetic yolk formation may be rare, whereas mixed auto- and heterosynthetic yolk formation may be common in the Echinodermata.

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Differences in Temperature Dependence of Early Development of Sea Urchins with Different Growing Seasons

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Abstract. Three species of sea urchin, *Hemicentrotus pulcherrimus, Anthocidaris crassispina,* and *Hemicentrotus depressus,* inhabit the tidal areas around the Misaki Marine Biological Station, on the Pacific coast of Japan. These sea urchins have different breeding seasons and hence their embryos show differences in temperature sensitivity. In this study, differences in the temperature dependence of their embryogenesis were examined. It was found that the upper and lower limits of the optimal temperature range for embryogenesis in each animal were species-specifically determined, and that the range of seawater temperature during the breeding season was within this optimal temperature range. A quasi-linear relationship was obtained between temperature and relative developmental velocity, which was defined as the ratio against the maximal velocity attained at the highest temperature within the optimal temperature range. Based on this result, a form of graphical analysis was devised to represent the differences in species-specific developmental temperature sensitivity. The physiological and ecological significance of species-specific temperature sensitivity was also discussed.

Introduction

Several reports have discussed the temperature dependence of early development of sea urchins (Loeb and Wasterneys, 1911; Koehler, 1912; Loeb and Chamberlain, 1915; Hörstadius, 1925; Needham, 1931; Tyler, 1936; Hoadley and Brill, 1937; Bougis, 1971; Stephens, 1972a, b; McEdward, 1985; Bosch *et al.*, 1987), amphibians (Hertwig, 1898; Ryan, 1941; Moore, 1942; Volpe, 1953a, b; Bachmann, 1969) and other animals such as an insect (Krogh, 1914a), copepods (Herzig, 1983a), and rotifers (Herzig, 1983b). Relationships between the rate of development of embryos and temperature during the breeding season have been reported only for amphibians in North America (Ryan, 1941; Moore, 1942; Volpe, 1953a, b; Bachmann, 1969).

I observed similar relationships in three sea urchin species living in the same locality. In the tidal and subtidal areas around the Misaki Marine Biological Station, Kanagawa Prefecture, on the Pacific coast of Japan (139°6′ E, 35°2′ N.L.), *Hemicentrotus pulcherrimus* spawns in winter to early spring (from January to April) when seawater temperature is between about 10° and 15°C. *Anthocidaris crassispina* spawns in summer (from July to early September) when the temperature is between about 20° and 27°C. *Pseudocentrotus depressus* spawns in autumn to early winter (from October to December) when the temperature is between about 13° and 23°C. Each seawater temperature range in each spawning season is optimal for the embryos of the respective sea urchin species. However, the relationship between temperature and the developmental rates of these three species has never been investigated. This report presents an analysis of the effect of temperature on the developmental rates of these three species of sea urchin, and discusses the ecological significance of the results.

Materials and Methods

The sea urchins, *Hemicentrotus pulcherrimus, Anthocidaris crassispina,* and *Pseudocentrotus depressus* were

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collected from the tidal area around the Misaki Marine Biological Station, Kanagawa Prefecture, on the Pacific coast of Japan (139°6′ E, 35°2′ N.L.). Experiments were always conducted in the peak spawning season for these sea urchins.

Eggs and sperm were obtained by introducing 0.5 *M* KCl into the body cavity. The eggs used were always obtained from only one mature individual to avoid mixing with eggs from premature or overmature individuals and to ensure optimal synchrony. The eggs were divided into groups and each group was inseminated after preincubation at a specific temperature, followed by culture with gentle stirring using a glass blade. To optimize developmental synchrony, all cultures were maintained in constant temperature water bathes adjusted with an accuracy of ±0.1°C as read with a standard thermometer.

Optimal temperature was defined as the temperature at which the developmental process from early cleavage to pluteus larva was able to proceed without any abnormal morphology. The upper and lower limits of the optimal temperature range for the development of each sea urchin species was experimentally determined by culturing embryos at temperatures increasing and decreasing at intervals of 1°C.

The times of the first, second, third, and fourth cleavage, hatching, mesenchyme ingression, and gastrulation, defined as the number of minutes after insemination when about 50% of the embryos had completed each stage, were measured. The size of the counted sample population was 50 to 60 embryos and the counting interval was 5 min for the first, second, third, and fourth cleavage, or 15 min for the stages later than hatching.

The reciprocal of the time required for embryos to develop into each stage was taken as the developmental velocity. The velocities were temperature-dependent, and maximal at the highest temperature within the optimal range temperature for each species. To clarify species-specific differences in the temperature dependence of developmental velocity, I introduced the relative developmental velocity defined as the ratio $(1/t_T)/(1/t_H)$, where t_T is the time required for embryos to develop into each stage at a specific temperature and t_H is the time required to develop into the respective stage at the highest temperature within the optimal temperature range.

Results

Hemicentrotus pulcherrimus embryos showed normal development within the temperature range of 5°C to 23°C. Normal development was shown by *Pseudocentrotus depressus* embryos within a range of 9°C to 25°C. Those of *Anthocidaris crassispina* showed normal development within a range of 16°C to 29°C. At 4°C, cleavage of *H. pulcherrimus* embryos was abnormal; the embryos were unable to form blastulae. The embryos showed no developmental change other than elevation of the fertilization membrane at temperatures lower than 3°C. At 15°C, *A. crassispina* embryos were unable to form normal plutei, at 14°C they were unable to develop into gastrulae, and at temperatures lower than 13°C they showed no blastula formation. The development of the embryos was nearly arrested at temperatures lower than about 10°C. At temperatures higher than the ranges optimal for normal development, the embryos of any species were unable to develop normally to the gastrula stage. Since the ranges of seawater temperatures during the spawning season are about 10°C to 15°C for *H. pulcherrimus*, about 13°C to 23°C for *P. depressus*, and about 20°C to 27°C for *A. crassispina* (Table I), it is apparent that the ranges of temperature optimal for normal embryogenesis of each species of sea urchin sufficiently cover those of seawater during the spawning season of each respective animal.

Within the ranges of temperature optimal for normal development of the sea urchin embryos, temperature had a significant effect on the velocity of development. Table II shows the times necessary for development of *H. pulcherrimus* into the first, second, third, and fourth cleavage stages, hatching, mesenchyme ingression, and

Table I

Range of optimal temperature for early development of three species of sea urchin, Hemicentrotus pulcherrimus, Pseudocentrotus depressus, *and* Anthocidaris crassispina, *and range of seawater temperature during their breeding seasons around tidal areas at the Misaki Marine Biological Station on the Pacific coast of Japan*

Species	Range of optimal temperature for early development (°C)	Range of seawater temperature during breeding season (°C)
Hemicentrotus pulcherrimus	5–23	10–17
Pseudocentrotus depressus	9–25	14–21
Anthocidaris crassispina	16–29	19–25

Optimal temperature for early development was defined as the temperature at which sea urchin embryos were able to develop into pluteus larvae without any abnormal morphology. Optimal temperature ranges were determined experimentally. Seawater temperature was measured at a depth of 1 m at a point in the tidal area around the Misaki Marine Biological Station. The temperature was taken once every week. The range of seawater temperature during the breeding season indicates the maximum fluctuation of the temperature, *i.e.*, the range between the highest and the lowest temperature during the season.

Table II

Effect of temperature on the times or the relative developmental velocity for Hemicentrotus pulcherrimus *embryos to arrive at each developmental stage*

	Temperature (°C)					
	5	10	12	15	2	23
Stage						
2-Cell	343 ± 6	162 ± 6	127 ± 2	90 ± 0	65 ± 0	60 ± 0
	(0.18 ± 0.01)	(0.37 ± 0.02)	(0.47 ± 0.01)	(0.67 ± 0.00)	(0.92 ± 0.00)	
4-Cell	513 ± 12	245 ± 15	188 ± 8	137 ± 2	97 ± 2	90 ± 0
	(0.18 ± 0.01)	(0.38 ± 0.01)	(0.49 ± 0.01)	(0.66 ± 0.01)	(0.93 ± 0.02)	
8-Cell	703 ± 12	323 ± 20	263 ± 26	192 ± 13	133 ± 5	122 ± 2
	(0.17 ± 0.01)	(0.38 ± 0.02)	(0.48 ± 0.01)	(0.66 ± 0.01)	(0.92 ± 0.00)	
16-Cell	888 ± 43	412 ± 28	330 ± 29	240 ± 10	170 ± 10	155 ± 5
	(0.18 ± 0.01)	(0.38 ± 0.01)	(0.48 ± 0.02)	(0.65 ± 0.01)	(0.93 ± 0.01)	
Hatching	2740 ± 348	1447 ± 105	1127 ± 88	865 ± 53	633 ± 21	620 ± 22
	(0.21 ± 0.01)	(0.43 ± 0.02)	(0.55 ± 0.02)	(0.72 ± 0.02)	(0.98 ± 0.00)	
Mesenchyme ingression	4150 ± 40	1800 ± 30	1440 ± 30	1010 ± 10	735 ± 5	665 ± 5
	(0.16 ± 0.00)	(0.37 ± 0.00)	(0.47 ± 0.01)	(0.66 ± 0.00)	(0.91 ± 0.00)	
Gastrulation	5035 ± 35	2170 ± 20	1755 ± 15	1250 ± 10	890 ± 10	815 ± 5
	(0.16 ± 0.00)	(0.38 ± 0.00)	(0.47 ± 0.01)	(0.65 ± 0.00)	(0.92 ± 0.01)	

Temperatures were adjusted to an accuracy of ±0.1°C. Numbers indicate times (minutes) taken for 50% of the embryos to arrive at each stage. Numbers in parentheses are relative velocities, the velocity at a given temperature being defined as the ratio of the reciprocal of the time required for development into each stage to the reciprocal of the time required to develop into the corresponding stage at the highest temperature. Each number is the average ± standard error for separate three experiments.

gastrulation at various temperatures within the ranges of temperature optimal for the embryos of this species. As it was easier to measure the times required for development into the first, second, and third cleavage stages and hatching more accurately than those of mesenchyme ingression, gastrulation, and subsequent stages, Table III lists only the times required for development into the first, second, and third cleavages and hatching for the other two species of sea urchin. The values in parentheses in Tables II and III indicate relative developmental velocities. It is obvious that at a specific temperature the relative velocities of all stages except hatching are nearly equal, and hence it can be said that the relative velocity is dependent only on temperature, and not on the developmental stage. Figure 1 presents the results shown in Table II in graph form, and it is apparent that a quasi-linear relationship exists between temperature and the relative velocity of every stage within each range of optimal temperature. In addition to this, the plots in Figure 1 overlap into a single quasi-linear curve, indicating that the relative velocities from fertilization to every stage except hatching can be plotted on such a curve. Extrapolation of the curve gives a temperature (3°C) at which the velocity of development is zero (T_0C).

It is noted that the quasi-linear curve designating the relationship between the relative velocity of hatching and temperature deviated from those of the other stages and temperatures. This deviation was observed in the embryos of the other species.

The relative velocities of development to the first, second, and third cleavages were then plotted on single curves for each of the three species of sea urchin, and the curves were found to be individually characteristic (Fig. 2). The data on which these curves were based are shown in Tables II and III. The differences between the curves indicate the differences in the relationships between temperature and velocity in the embryos of *H. pulcherrimus*, *P. depressus*, and *A. crassispina*. These differences clearly reflect the differences in the optimal range of temperature for embryos of each species of sea urchin.

The differences in temperature dependence of their early development are thus indicated by the differences in the positions of the curves in the coordinates of temperature and relative developmental velocity. It should also be noted that within the range of seawater temperatures during the spawning season, the range of relative developmental velocity was nearly constant (from about 0.4 to 0.8), regardless of the species of sea urchin.

Discussion

Temperature is a primary limiting factors affecting a wide range of biological phenomena—from metabolic biochemical reactions to the geographical distributions

Table III

Effect of temperature on the times or the relative developmental velocity for Anthocidaris crassispina *and* Pseudocentrotus depressus *embryos to arrive at each developmental stage*

	Temperature (°C)				
Anthocidaris crassispina	16	20	23	26	29
Stage					
2-Cell	178 ± 3	105 ± 5	65 ± 0	50 ± 0	40 ± 0
	(0.23 ± 0.01)	(0.38 ± 0.02)	(0.62 ± 0.00)	(0.80 ± 0.00)	
4-Cell	293 ± 7	168 ± 7	105 ± 5	80 ± 5	60 ± 0
	(0.20 ± 0.01)	(0.38 ± 0.01)	(0.63 ± 0.02)	(0.81 ± 0.01)	
8-Cell	405 ± 15	230 ± 10	145 ± 5	113 ± 3	93 ± 2
	(0.23 ± 0.01)	(0.41 ± 0.01)	(0.64 ± 0.01)	(0.83 ± 0.01)	
16-Cell	520 ± 20	290 ± 10	183 ± 2	137 ± 3	113 ± 2
	(0.22 ± 0.01)	(0.39 ± 0.01)	(0.61 ± 0.01)	(0.82 ± 0.01)	
Hatching	1130 ± 100	715 ± 65	513 ± 38	420 ± 30	355 ± 25
	(0.31 ± 0.01)	(0.50 ± 0.01)	(0.69 ± 0.01)	(0.85 ± 0.01)	

	Temperature (°C)				
Pseudocentrotus depressus	10	15.5	20	23	25
Stage					
2-Cell	265 ± 5	128 ± 3	88 ± 3	75 ± 0	70 ± 0
	(0.27 ± 0.01)	(0.56 ± 0.01)	(0.79 ± 0.01)	(0.93 ± 0.00)	
4-Cell	385 ± 5	195 ± 5	128 ± 3	108 ± 3	103 ± 3
	(0.27 ± 0.01)	(0.53 ± 0.00)	(0.81 ± 0.01)	(0.95 ± 0.00)	
8-Cell	500 ± 20	255 ± 5	173 ± 3	143 ± 3	138 ± 3
	(0.28 ± 0.01)	(0.54 ± 0.01)	(0.80 ± 0.01)	(0.93 ± 0.00)	
16-Cell	610 ± 30	318 ± 8	215 ± 5	178 ± 3	168 ± 3
	(0.28 ± 0.01)	(0.53 ± 0.01)	(0.78 ± 0.01)	(0.94 ± 0.00)	
Hatching	1770 ± 30	975 ± 15	655 ± 5	538 ± 8	455 ± 5
	(0.26 ± 0.01)	(0.47 ± 0.01)	(0.70 ± 0.01)	(0.85 ± 0.01)	

Legend is same as for Table II. Each number is the average ± standard error for two separate experiments.

of species. Of course, development is also dependent on temperature. Some early embryologists tried to determine whether the Arrhenius law could be applied to developmental velocity. There was even a report claiming that the law indeed held for the relationship between temperature and developmental velocity (Crozier, 1926), but contrary to this contention, it has since become evident that the law can hardly be applied to this relationship. Instead, it has been proved that the relationship can be represented as a sigmoidal curve, the major part of which is linear (Loeb and Wasterneys, 1911; Krogh, 1914b; Loeb and Chamberlain, 1915; Atlas, 1935; Tyler, 1936; Hoadley and Brill, 1937; Ryan, 1941; Volpe, 1953a, b; Bachmann, 1969; Stephens, 1972a). This is also confirmed in the present work. Despite attempts by several authors to analyze these relationships graphically (Krogh, 1914a, b; Crozier, 1926; Bělehrádek, 1930; Atlas, 1935; Tyler, 1936; Bachmann, 1969), no clear, sharp, and precise analysis has yet been achieved.

In addition, except for the stage of hatching, the curves representing the relationship were confirmed to be nearly the same in the present experiment, regardless of the developmental stages of the sea urchins used. This phenomenon was first noticed by Atlas in the embryogenesis of *Rana pipiens* (Atlas, 1935), and was also found to be true when I re-examined previously published data on the early development of *Arbacia punctulata* (Loeb and Wasterneys, 1911; Loeb and Chamberlain, 1915; Hoadley and Brill, 1937), *Strongylocentrotus droebachiensis* (Stephens, 1972a), and *Dendraster excentricus* (Tyler, 1936). Hence, this phenomenon seems to be universally valid for early development of animals of any kind, within the range of optimal temperature. Thus it can be regarded as an embryological law.

It is noteworthy that the curve indicating the relationship between the relative velocity of hatching and temperature deviated. The reason for this deviation is unknown, but it should be pointed out here that, in comparison with cleavage and morphogenetic movement, hatching is a relatively simple biochemical process, *i.e.,*

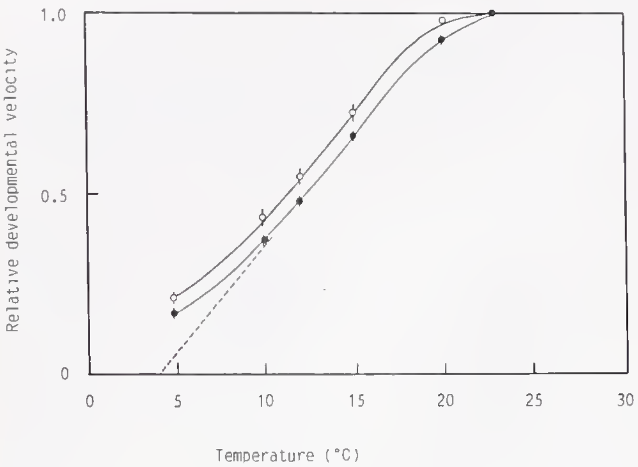

Figure 1. Relationship between temperature and the relative developmental velocity of *Hemicentrotus pulcherrimus* embryos.

The results are based on the data shown in Table II. Each solid circle and bar represents the mean and standard error respectively, of the relative velocities for development into the first, second, third, and fourth cleavages, mesenchyme ingression, and gastrula stage. Each open circle and bar indicates respectively the mean and standard error of the relative velocity for development into the hatching stage at a given temperature. The dotted line is an extrapolation of the curve, assuming it to be a straight line.

exocytosis of hatching enzyme and its hydrolytic action on the fertilization membrane.

The factors determining the temperature tolerance or dependence of embryos have not yet been elucidated. Bělehrádek (1930) proposed that in general biological processes including development protoplasmic viscosity is the main factor determining the effect of temperature. Stephens (1972b) has tried to explain the temperature tolerance of embryos in terms of Inoué's mitotic dynamic theory. Cellular activities in early embryogenesis, such as cleavage and cell adhesion (Fujisawa and Amemiya, 1988) are significantly affected by temperature, and these processes are mainly dependent upon cytoskeletal activities. However, cytoskeletal elements do not seem to be a candidate for a factor, since actin, as well as tubulin, is reported to be evolutionarily stable (Loewy, 1952; Hatano and Oosawa, 1966; Adelman and Taylor, 1969; Luduena and Woodward, 1973; Krauhs *et al.*, 1981; Hall *et al.*, 1983; Sullivan and Cleveland, 1984). Temperature sensitivities of enzymes of DNA synthesis may also be another important factor since mitosis is closely connected with DNA synthesis. It is noteworthy that the temperature optimum of DNA polymerase in *Strongylocentrotus purpuratus* and *Lytechinus pictus* embryos is 30°C (Loeb, 1969) as well as of DNA topoisomerase (Poccia *et al.*, 1978).

Control of the timing mechanism of gametogenesis in response to temperature is another important problem that would be useful to elucidate, since this mechanism is directly related to embryogenesis and hence affects the proliferation of animals as well as the distribution of mature adults. There have been several reports on optimal temperature response of gametogenesis (Barnes, 1963; Fries, 1964; Meats and Khoo, 1976; Shrode and Gerking, 1977). However, this problem is beyond the scope of the present work.

The ecological significance of the temperature dependence of development has been investigated only in amphibians of North America (Ryan, 1941; Moore, 1942; Volpe, 1953a, b; Bachmann, 1969), but the present results confirm the ecological significance of temperature dependence of this phenomenon. Fortunately, three types of ecological elements, *H. pulcherrimus*, a cold-temperate element, *P. depressus*, a medium-temperate element, and *A. crassispina*, warm-temperate elements, were found to coexist in the tidal and subtidal area around the coast near the Misaki Marine Laboratory. This area is the southern limit for *H. pulcherrimus* while it is the northern limit for *A. crassispina*, and thus it seems reasonable that the embryos of *H. pulcherrimus* should develop in winter while those of *A. crassispina* develop in summer. Temperature tolerance seems to be narrower and stricter in the embryos than in the adults of these species, and this tolerance characteristic is thought to determine the distributions of the animals.

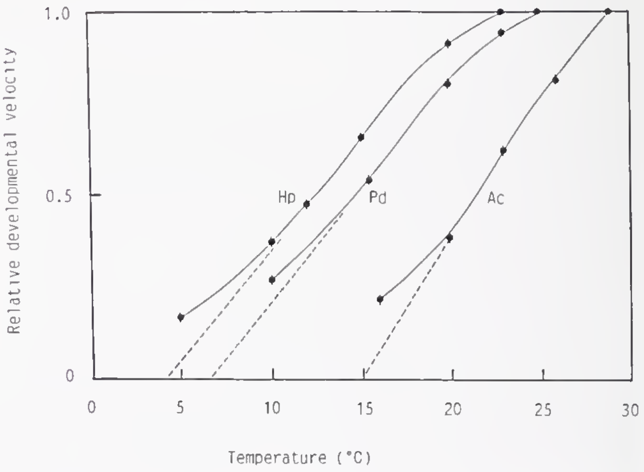

Figure 2. Relationship between temperature and the relative developmental velocity of the three species of sea urchin, *Hemicentrotus pulcherrimus*, *Anthocidaris crassispina*, and *Pseudocentrotus depressus*.

The results are based on the data in Tables II and III. Each value represents the average ± standard error of the relative velocities for development into the first, second, third and fourth cleavage stages at a given temperature. Hp, *Hemicentrotus pulcherrimus*; Pd, *Pseudocentrotus depressus*; Ac, *Anthocidaris crassispina*.

Diadema setosum may be another example. This sea urchin lives widely in the Indo-Pacific sea and spawns throughout the year in the tropics where seawater temperatures are nearly always above 25°C (Mortensen, 1937, 1938; Pearse, 1974). In Sagami Bay and neighboring Suruga Bay on the south coast of Japan, the northern limit of the distribution of this species, the animal spawns once a year, *i.e.*, on the days of full moon in August (Yoshida, 1952). I am now attempting to clarify whether the temperature tolerance hypothesis holds for this species of sea urchin. Similar examples may also be found in *Arbacia punctulata* and *Strongyrocentrotus droebachiensis*, which live in Cape Cod Bay. Based on data from Loeb and Wasterneys (1911), Loeb and Chamberlain (1915), Hoadley and Brill (1937), and Stephens (1972a), clear correlations seem to be established between the spawning seasons of these species, seawater temperature, and the temperature tolerance of their embryos. Various species of sea urchin have a wide range of distribution: from equatorial to arctic and antarctic seas, and from shallow to deep seas. It is hoped that investigators will soon confirm whether the hypothesis holds globally for these sea urchins.

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Developmental Variability (Pelagic and Benthic) in *Haminoea callidegenita* (Opisthobranchia: Cephalaspidea) is Influenced by Egg Mass Jelly

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Abstract. *Haminoea callidegenita* Gibson and Chia (1989) has a pattern of development similar to that of other lecithotrophic opisthobranchs, except for the stage at hatching. In this species, both veligers and juveniles hatch from each egg mass. The percent of each hatching stage was variable among masses, with most masses having 30 to 50% of total hatchlings emerging as veligers. Both veligers and juveniles emerge throughout the entire hatching period (lasting 3–11 days), although the percentage of veligers decreases during this period. Encapsulated embryos cultured without egg mass jelly had approximately 80% of the total hatchlings emerging as veligers. Separated embryos cultured in the presence of egg mass jelly pieces hatched with percentages of veligers similar to that observed in intact egg masses, suggesting the possibility of a diffusible compound present in the jelly mass that induces intracapsular metamorphosis. Egg mass jelly also induces metamorphosis in hatched veligers. A juvenile and adult food source (the green alga *Chaetomorpha linum*) induces extracapsular metamorphosis only.

Introduction

Opisthobranch molluscs are hermaphroditic with internal fertilization (Morton, 1979). As spawning occurs, fertilized eggs are enclosed either individually or in groups by membranous capsules that are then linked together to form strings within a gelatinous mass (Purchon, 1977). The development of a number of opisthobranch species has been described (Thompson, 1958, 1967; Rao, 1961; Smith, 1967; Chia, 1971; Bridges, 1975; Switzer-Dunlap and Hadfield, 1977; Bickell, 1978; Chia and Koss, 1978; Clark *et al.*, 1979; Williams, 1980; others). In all opisthobranch species that have been studied, there is an early phase of encapsulated development (benthic) followed by a veliger phase falling into one of three categories (Thompson, 1967): planktotrophic (feeding, pelagic veliger), lecithotrophic (non-feeding, pelagic veliger), and direct development (metamorphosis occurs within the egg mass, with the veliger stage either present or suppressed; Bonar, 1978).

Although it has been thought that each opisthobranch species has one type of development, at least a half-dozen species appear to be poecilogonous (reviewed by Bonar, 1978). The validity of poecilogony in these and other marine invertebrates is currently under discussion (Hoagland and Robertson, 1988). In opisthobranchs, developmental patterns may vary geographically (*Tenellia pallida* Alder and Hancock, Eyster 1979; *Elysia chlorotica* Gould, West *et al.*, 1984), with changing environmental conditions (such as food availability as in *Spurilla neapolitana*, Clark and Goetzfried, 1978), and simultaneously among individuals of one population (*Tenellia pallida*, Eyster, 1979).

Haminoea callidegenita is a cephalaspidean opisthobranch found in the San Juan Islands, Washington State. *H. callidegenita* has an unusual form of development which includes the hatching of both lecithotrophic veligers and juveniles from the same egg mass. In this paper, we describe it's larval development with emphasis on the hatching stage. Preliminary results indicate that the egg mass jelly contains a diffusible compound that promotes intracapsular metamorphosis.

Materials and Methods

Collection and maintenance

Adults and egg masses of *Haminoea callidegenita* were collected from Spencer's Spit, Lopez Island, Washing-

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ton, from June 1985 to April 1987. Spencer's Spit is a shallow lagoon (<1 m) with limited access to open water, influenced slightly by tidal cycles, and subject to wide fluctuations in temperature. The lagoon has a silty substratum and is dominated by the green alga *Chaetomorpha linum* (O. F. Mull.) Kutz and *Ulva* sp.

Adults and egg masses were maintained at Bamfield Marine Station, Bamfield, British Columbia (June 1985 to August 1986) and at the Department of Zoology, University of Alberta, Edmonton, Alberta, Canada (September 1986 to April 1987). Adults were maintained in glass aquaria with a continuous flow of seawater at ambient temperatures (6–12°C), and were supplied with *Chaetomorpha linum* and *Ulva* sp. as food. Egg masses were collected immediately after spawning and maintained in Pyrex dishes at either ambient seawater temperatures or 14–15°C. Culture water was replaced daily with bag-filtered (1 μm) seawater. Observations made on egg masses that were spawned in the laboratory were compared with those collected in Spencer's Spit. Newly hatched veligers and juveniles were removed daily and cultured as outlined for egg masses, with the addition of *Chaetomorpha* and *Ulva* as food in the case of the juveniles. Flakes of cetyl alcohol were added to reduce surface tension, thus decreasing the rate at which larval shells became trapped at the surface (Hurst, 1967). Antibiotics were not generally used. Cultures of veligers and/or juveniles were cleaned every second day. When juveniles reached approximately 3 mm in length (measured while they were actively crawling) they were transferred to partially submersed 500 ml beakers with vents of 335 μm Nitex mesh to provide a gentle but continuous flow of seawater, at temperatures of 12–15°C.

Light microscopic observations and measurements of embryonic, larval, and early juvenile stages were made using a calibrated ocular micrometer on a Reichert compound microscope or a Wild M5 microscope. Photomicrographs were taken using a Wild Photoautomat MPS 45 camera on a Wild compound microscope.

Induction of intracapsular metamorphosis

Developmental stage at hatching was determined by examining encapsulated embryos cultured: (1) within intact egg masses (n = 9 egg masses); (2) within egg masses sliced into approximately 2 mm thick segments (n = 10 slices, each from a different egg mass); and, (3) completely separated from the egg mass, with all remnants of the jelly mass removed; n = 10 cultures from different egg masses). The jelly layers were easily peeled away from the egg strings which were then opened to release the encapsulated embryos. The capsules were rinsed in filtered seawater to remove any possible remnants of jelly. Separated embryos were cultured under three conditions: (a) embryos only (n = 10 cultures, each containing embryos from a different egg mass); (b) embryos with *Chaetomorpha* (2 mg blotted wet weight *Chaetomorpha*/40 ml seawater, or 0.05 mg/ml; n = 10); and, (c) embryos with pieces of egg mass jelly floating in the culture water, but not in direct contact with the embryos (14 mg blotted wet weight jelly/40 ml seawater, or 0.35 mg/ml; n = 10). Embryos were cultured from early cleavage stages (0–2 days after oviposition) until hatching occurred (32–39 days after oviposition). Egg masses used were all produced by different females. All cultures were maintained at 14–15°C.

Sample sizes indicated above (n) represent the number of cultures per condition. Intact egg masses and slices were cultured individually per 100 ml jar. Separated embryos were maintained at densities of 50–60/jar. All jars contained 40 ml of bag filtered seawater. All cultures were cleaned every second day and maintained under the specified conditions until hatching occurred (approximately 32 days after oviposition). During the hatching period, each culture was examined daily and new hatchlings removed and counted.

Induction of extracapsular metamorphosis

Potential extracapsular metamorphic inducers were examined by providing veligers hatched from intact egg masses with one of several substrata on the day of hatching. Culture dishes (10 × 50 mm plastic petri dishes) were treated with Clorox (5% sodium hypochlorite) for 2 h, then triply rinsed and soaked in distilled water. Substrata provided were: (1) bag-filtered (1 μm) seawater only (control; n = 12 cultures); (2) filtered seawater plus *Chaetomorpha* (n = 7); (3) filtered seawater plus surface sediment (n = 7); and, (4) filtered seawater plus pieces of egg mass jelly (n = 15). Each culture dish contained five veligers. Observations were made at 12-h intervals until metamorphosis occurred or the veligers died (up to a maximum of 20 days after hatching). Culture dishes were not treated with Chlorox after the onset of the experiment. All cultures were maintained at approximately 20°C, a temperature commonly encountered during the summer months in the shallow lagoon at Spencer's Spit. All substrata were collected from the adult habitat.

Results

Oviposition and development

Adult *Haminoea callidegenita* were found in Spencer's Spit throughout the year. Viable egg masses were collected in all months except January and December. *H. callidegenita* egg masses were similar to Hurst's (1967) type D opisthobranch egg mass. They were short, thick cylinders, ranging from almost ovate (10 × 5 × 5 mm) to sausage shaped (36 × 6 × 6 mm). A typical mass produced by a 26 mm adult was 15 × 5 × 5 mm and

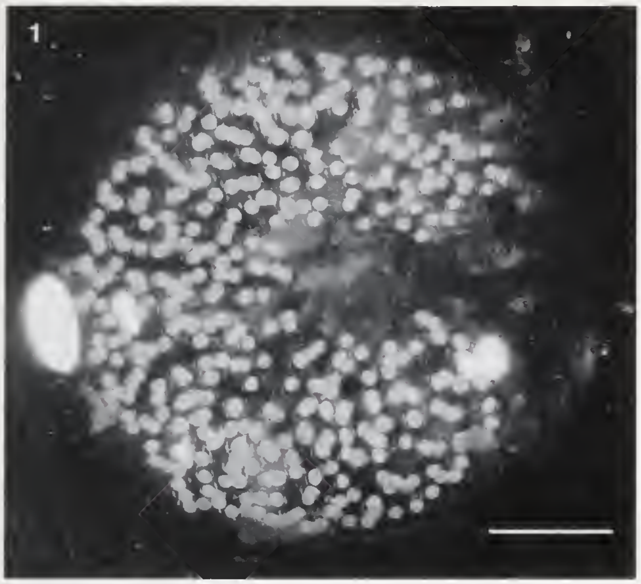

Figure 1. Photograph of a *Haminoea callidegenita* egg mass. Scale = 2 mm.

took approximately 20 min to be deposited. Egg masses were attached to the substrate along the length of one side, and were slightly curved in shape (Fig. 1), a result of the adult turning slightly towards the mass during oviposition. Adults did not show any substrate preference for oviposition and deposited masses on any available solid surface, both in the field and under laboratory conditions.

The bright orange-yellow eggs were individually encapsulated and arranged in a continuous string. This string spiralled through the periphery of the egg mass (irregularly deposited at a depth of approximately 1.5 mm) leaving the center and outer portion of the jelly free of embryos. Egg masses contained an average of 21 eggs/mm^3 egg mass (n = 10 masses), for a total of 200 to 700 eggs per mass. Density of eggs was variable within a single mass, as well as among masses. Uncleaved eggs measured 230 × 210 μm and were encased in a 410 × 330 μm capsule. Development appeared to be synchronous within the mass until approximately 30–32 days after oviposition. Cleavage, gastrulation, and development of the veliger follow the same pattern as described in *Haloa japonica* Pilsbry (Usuki, 1966), a closely related species with lecithotrophic development. The chronology of major developmental events is summarized in Table I and illustrated in Figures 2–6. Well developed veligers, with lengthened propodia, rhythmic contractions of the heart, and capable of complete retraction into the shell are visible in the egg masses 29 days after oviposition (Fig. 3). Hatching began approximately 3 days later. Some individuals from each egg mass hatched as veligers (Fig. 4), but the majority metamorphosed within the egg capsule (Fig. 5) and hatched as juveniles.

A few days before the onset of hatching, the jelly mass began to deteriorate and was colonized by diatoms and nematodes, as has been described for opisthobranch egg masses (Davis, 1967). Hatching generally occurred over a period lasting from 3 to 11 days. At hatching, capsule walls softened (*i.e.*, became flexible and readily distorted) and were eventually split by the larval propodium, allowing the individual to escape. This process took approximately 1.5 to 2 min after the first visible softening of the capsule. After the larva emerged from the capsule, it slowly worked its way out of the jelly mass. Veligers would then swim away, while juveniles often remained on the deteriorating mass or crawled to filaments of *Chaetomorpha* if available. Hatching of both veligers and juveniles occurred in the same way.

Hatched veligers did not grow during the pelagic period: shells remained 360 ± 16 × 280 ± 10 μm in size (veliger length 420 μm including extended velum) throughout the pelagic period (measured as by Hurst, 1967). Morphological changes other than decreasing yolk reserves were not visible. Neither the presence of

Table I

Summary of developmental events in Haminoea callidegenita *for egg masses cultured at 15°C*

Time (h, days)	Developmental event
0 h	Oviposition
24 h	First cleavage (holoblastic and equal)
46 h	Second cleavage
6 days	Blastula
7–8 days	Gastrula
10 days	Cephalopedal rudiment visible
12 days	Cilia and shell material appear, embryos irregularly rotating in capsules
13 days	Foot and velar lobes separate, shell growth over posterior quarter of yolk mass, regular rotation of embryos in capsules
14 days	Velum bilobed, cilia capable of beating metachronically
15 days	Operculum beginning to appear, ciliary beating can be arrested for short periods
16 days	Operculum present, shell growth to base of cephalopedal rudiment and separate from digestive glands, embryos rotating slowly (filling most of the capsules) and can change direction of rotation
18 days	Statocysts visible, 2 digestive glands distinct, shell curved 90° to larval right, body whorl of shell rounded
20 days	Eyes visible
21 days	Appearance of propodium
22 days	Esophagus and intestine visible, flexion of velar lobes, propodial thickening visible
25 days	Stomach visible, heart coelescing, complete retraction of larvae if disturbed by bright lights or water movements
29 days	Heart contracting, anus visible
32–39 days	Metamorphosis and/or hatching

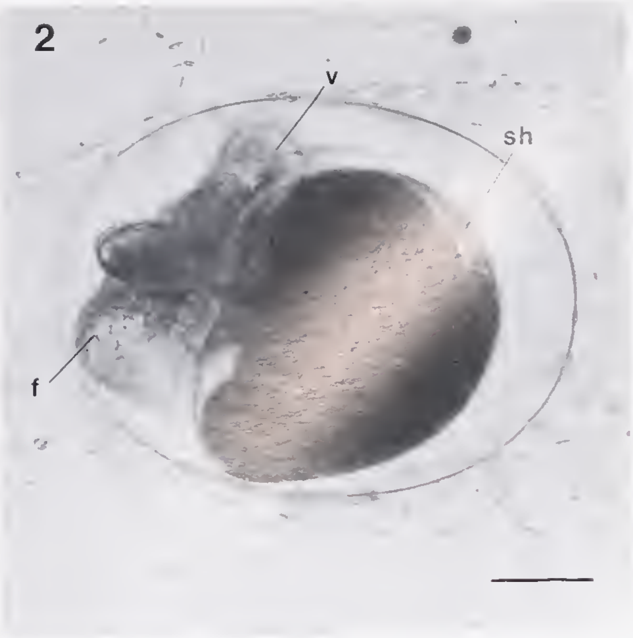

Figure 2. Photomicrograph of a 16-day *Haminoea callidegenita* veliger, also showing diatoms in the egg mass jelly (15°C). Scale = 90 μm. Legend: f-foot; sh-shell; v-velum.

unicellular algae in the gut nor the uptake of pigment by the digestive gland were evident, indicating that these veligers did not ingest food particles, as is known to occur in some other lecithotrophic veligers (Thompson, 1958; Kempf and Hadfield, 1985). *H. callidegenita* veligers were poor swimmers in culture conditions and periodically rested on algae and the bottom of the culture dish. Some veligers metamorphosed within 24 h of hatching, others remained pelagic for up to 20 days, when they successfully completed metamorphosis (5% mortality).

The majority of veligers metamorphosed within the capsule and hatched as juveniles. At metamorphosis, velar cilia were lost, the propodium elongated anteriorly, and the velar lobes gradually resorbed (Fig. 5). The larval shell was retained. Hatching occurred as described for extracapsular metamorphosis. Within 24 h of hatching, the foot lengthened posteriorly, and the operculum was cast off. As the remnants of the velar lobes were gradually resorbed, the cephalic lobe buds began to form on the posterior part of the head. Also, the orientation of the shell began to shift towards that of the adult, so the center of the aperture became positioned slightly to the right of the head.

Metamorphosis of individuals hatched as veligers occurred as described for intracapsular metamorphosis. Veliger and juvenile morphologies were the same for both encapsulated and hatched veligers.

All juveniles had sufficient (although decreasing) yolk reserves to allow several days delay in the onset of feeding. If food was available, juveniles usually began feeding within 24 h of metamorphosis, as indicated by the digestive glands becoming brown. Juveniles were epiphytic and particle grazers as were the adults. The buccal mass, radula, salivary glands, gizzard, and gizzard plates were visible and functional 4 days post-metamorphosis. Feeding setae, as described by Berrill (1931) in *H. hydatis* Linne, were not observed. Newly metamorphosed juveniles had resorbed most of the velar lobes and flattened the foot to extend anteriorly to project slightly in front of the head and posteriorly to the edge of the aperture. Rapid shell growth on the left side of the juvenile projected the shell posteriorly along the aperture. Thus, the overall shape changed from the veliger coil to a longer shell with an elongate aperture (Fig. 6). Growth of the foot was evident along the flaring of the aperture and, approximately 10 days after metamorphosis, the posterior pallial complex began to project beyond the body whorl of the shell. Animals become sexually mature when approximately 13 mm in length.

Induction of intracapsular metamorphosis

There was considerable variation in the relative proportions of veligers and juveniles hatching from egg masses cultured under identical conditions (Fig. 7). The percentage of total hatchlings (all hatchlings released from one egg mass throughout the entire hatching period) that emerged as veligers varied from 9.02% to 71.57% in 9 egg masses, although most masses fell within the 30–50% range. The mass with the smallest veliger component (9%; mass #5 in Fig. 7) was a medium sized mass (approximately 400 hatchlings) and hatching occurred over a relatively long period of time (10 days). The mass with the largest veliger component (70%; mass #7 in Fig. 7) was a large mass (approximately 600 hatchlings) and hatching occurred over a short period (3 days). Only one other mass had a 3-day hatching period (mass #6 in Fig. 7); approximately 40% of the hatchlings emerged as veligers from this mass. Mass #6 was also very small (150 hatchlings).

From each egg mass, hatching occurred over several days (Fig. 7). The relative proportions of veligers and juveniles changed throughout this period, with the percentage of veligers decreasing (Fig. 8) and the percentage of juveniles increasing with time, as would be expected as the juveniles represent a later stage of development. However, all egg masses had some juveniles emerging on the first day of hatching and some veligers hatching throughout most of the hatching period. Our estimation of the juvenile component is probably slightly high as observations were made every 24 h, and hatched veligers have been observed to metamorphose within this time interval. Despite a few days variation in the onset of hatching among masses, the same distribution of hatching stages was evident among individual masses.

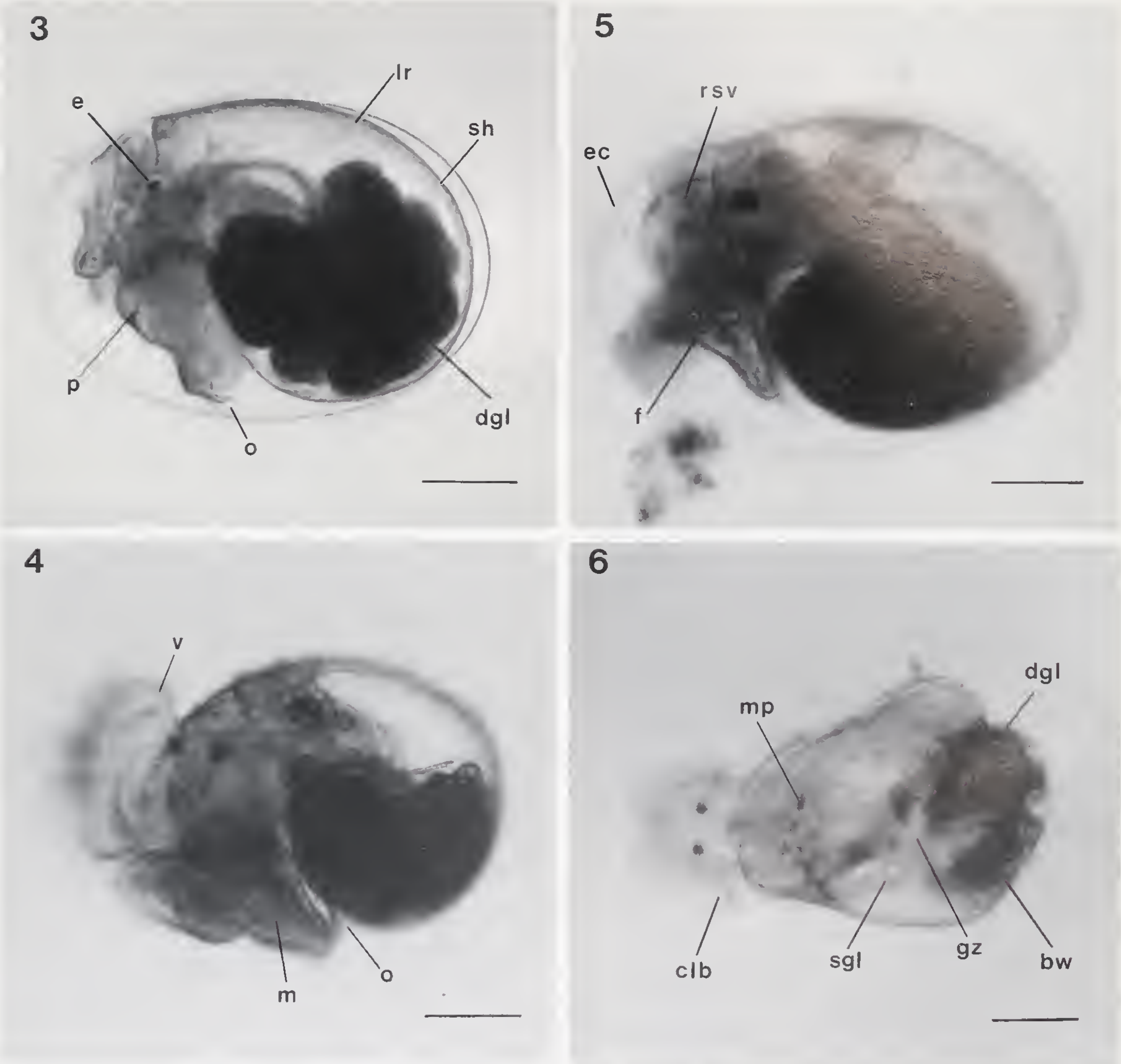

Figure 3–6. Photomicrographs of *Haminoea callidegenita* veligers and juveniles. Age is listed in days (d) for animals cultured at 15°C: (3) veliger, 29 d, scale = 100 μm; (4) veliger, 1 d hatched (31 d after oviposition), scale = 100 μm; (5) encapsulated juvenile, 32 d, scale = 100 μm; (6) juvenile, 10 d post-metamorphosis, scale = 300 μm. Legend: bw-body whorl of shell; clb-cephalic lobe bud; dgl-digestive gland; e-eye; ec-embryonic capsule; f-foot; g-gizzard; lr-larval retractor muscle; m-metapodium; mp-mantle pigment; o-operculum; p-propodium; rsv-partially resorbed velum; sgl-salivary gland; sh-shell; v-velum.

It is possible that in opisthobranch egg masses, the jelly may influence oxygen diffusion so that O_2 tension is lower in the center of an egg mass than at the periphery, thus slowing the rate of development of the central embryos. As *H. callidegenita* produce thick, cylindrical egg masses, and as the egg string is irregularly deposited in each jelly mass, we thought the distance between embryo and egg mass surface may be influential in determining the rate of development and therefore the hatching stage. The effect of decreasing the jelly thickness around developing embryos was determined by examining the percent veligers hatching daily from intact egg masses, masses cut in 2-mm thick slices, and encapsulated embryos removed from the mass jelly. Encapsulated embryos could be raised successfully without the jelly mass; there was a 100% success rate in hatching of healthy veli-

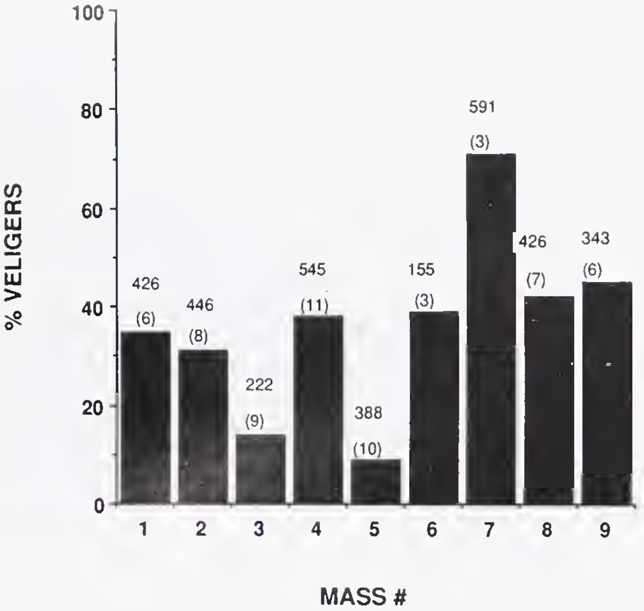

Figure 7. Hatching in *Haminoea callidegenita* egg masses: values plotted are the percent of total hatchlings per mass that emerged as veligers (n = 9 egg masses; 15°C). Numbers above each bar are total number of hatchlings per mass (top number) and duration of the hatching period in days (in brackets).

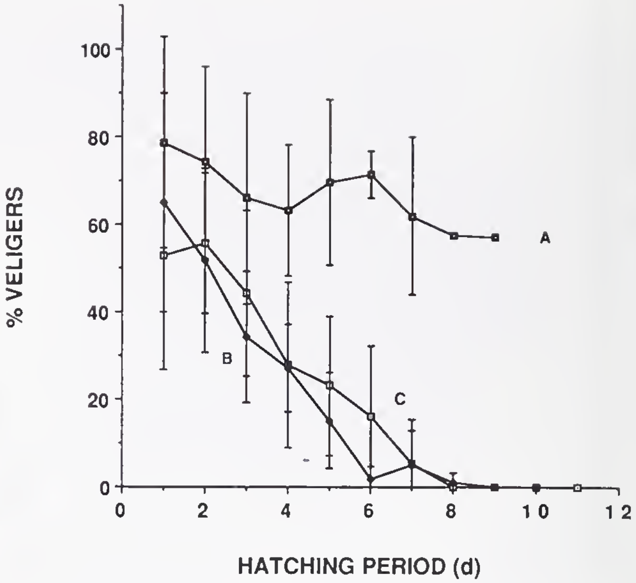

Figure 9. Effects on the hatching stage of removing egg mass jelly from *Haminoea callidegenita* embryos. Values plotted are means plus standard error bars for the percent daily hatchlings that emerged as veligers on each day of the hatching period, for three treatment groups (15°C): (A) embryos separated from the egg mass (n = 10 cultures); (B) egg mass slices (n = 10); and, (C) entire egg masses (n = 9).

gers and juveniles, which proceeded to metamorphose (in the case of the hatched veligers) and grow at the expected laboratory rate (0.02 mm/day). Intact egg masses and mass slices showed approximately the same percentage of veligers released throughout the hatching period (Fig. 9). Veligers represented 60% of the hatchlings at the onset of hatching (day 1), decreasing to 5% on day 7, and 0% by the end of the hatching period (*i.e.*, all hatchlings emerged as juveniles on the last day of hatching). Veligers hatching from capsules separated from the egg mass jelly represented approximately 80% of the hatchlings at the onset of hatching, and this rate remained relatively consistent throughout the hatching period, decreasing to 65% on the last day of hatching (day 9).

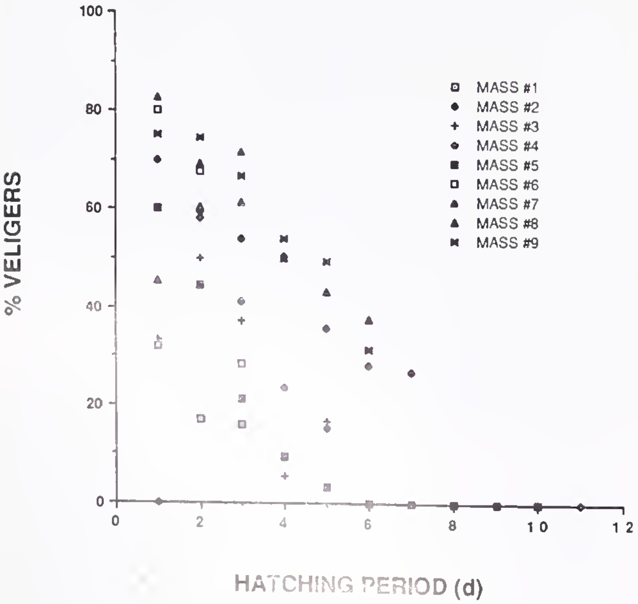

Figure 8. Distribution of developmental stages at hatching in *Haminoea callidegenita* plotted as the percent daily hatchlings that emerged as veligers throughout the hatching period (time in days; 15°C). Each symbol represents a different egg mass (n = 9 egg masses).

Embryos separated from the egg mass jelly were placed under three culture conditions: control (seawater only), seawater plus *Chaetomorpha*, and seawater plus pieces of egg mass jelly. Isolated embryos showed a distribution of hatching stages similar to that of the previous experiment (Fig. 10): 95% of the hatchlings were veligers on the first day of hatching, decreasing to 75% on the last day (day 5). Embryos cultured in the presence of *Chaetomorpha* showed similar results (73% released as veligers on day 1 of hatching, and 85% on the last day, day 4). However, embryos cultured in water containing egg mass jelly followed the same pattern as did hatchlings released from intact egg masses: 72% hatched as veligers on day 1, decreasing to 0% on day 6, the last day of the hatching period.

Induction of extracapsular metamorphosis

Extracapsular metamorphosis was examined in veligers which hatched from intact egg masses to see if the

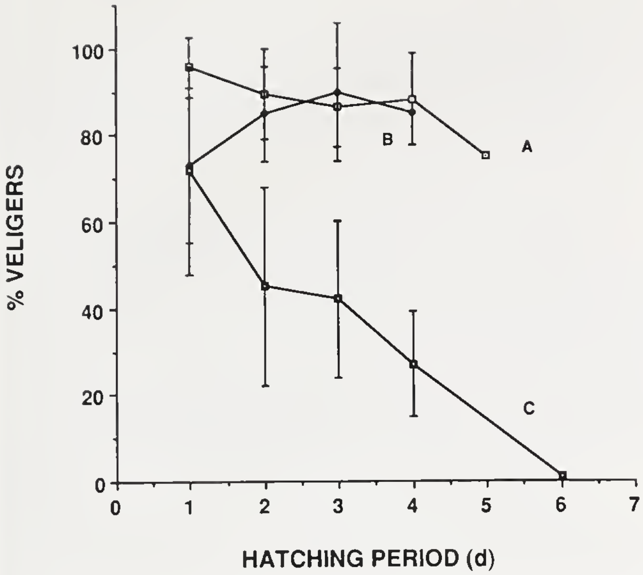

Figure 10. Effects of culturing separated embryos in the presence of: (A) seawater only (n = 10 cultures); (B) seawater plus *Chaetomorpha linum* (n = 10); and (C) seawater plus egg mass jelly pieces (n = 10). Values plotted are means plus standard error bars for each day of the hatching period.

rate of metamorphosis could be enhanced by substrata collected from the adult habitat. Three substrata were tested, all of which were frequently observed in association with small juveniles in Spencer's Spit: *Chaetomorpha*, silt, and egg mass jelly. Metamorphosis occurred under all of these conditions (Fig. 11). The presence of *Chaetomorpha* and egg mass jelly enhanced the rate of metamorphosis such that it took 9 days for all veligers to successfully metamorphose (2.90% and 0.00% mortality, respectively), rather than 20 days as in the control conditions (5.00% mortality). Veligers provided with silt as a substratum showed 5.70% mortality.

Discussion

The development of *Haminoea callidegenita* is morphologically similar to that described for *Haloa japonica* (Usuki, 1966), another cephalaspidean species, and to lecithotrophic opisthobranchs in general (Thompson, 1967). However, the timing of metamorphosis in *H. callidegenita* is variable: veligers can metamorphose within the egg capsule or after hatching. This unusual pattern has the adaptive advantage of allowing immediate recruitment within a population while simultaneously allowing larval dispersal. However, the prominence of pelagic veligers of this species in its natural habitat has not yet been determined (Gibson and Chia, in progress).

The percent of total hatchlings that emerged as veligers was variable among masses cultured simultaneously under identical conditions. Although mass size (ranging from 155 to 591 hatchlings/mass) and duration of the hatching period (from 3 to 11 days) may have influenced the percentage of veligers hatching from a particular mass, it seemed that there was a greater variance than could be accounted for by only these two components. Each of the masses examined were produced by different individuals; whether the observed variance is genetically influenced has not yet been examined.

The thickness of the jelly layer surrounding the embryos did not affect hatching stage (whole *vs.* sliced masses). However, embryos that were isolated from the egg mass jelly showed a high percentage of individuals hatching as veligers, and throughout the hatching period this percentage did not decrease as much as would be expected from a comparison with percentages obtained from intact egg masses. In addition, culturing embryos in the presence of mass jelly produced percentages of intra- and extracapsular metamorphosis as seen in intact egg masses. This suggests that either the egg mass jelly or something associated with the jelly promotes intracapsular metamorphosis of some, but not all, individuals. Direct contact between capsule and egg mass jelly is not required to induce metamorphosis, indicating that the inducing factor is a diffusible chemical. However, the chemical nature of this compound is yet to be determined (Gibson and Chia, in progress).

Chaetomorpha linum provided the surface most commonly grazed for epiphytes by *H. callidegenita* juveniles and adults. The presence of *Chaetomorpha* did not increase the rate of intracapsular metamorphosis. However, the rate of metamorphosis of individuals that

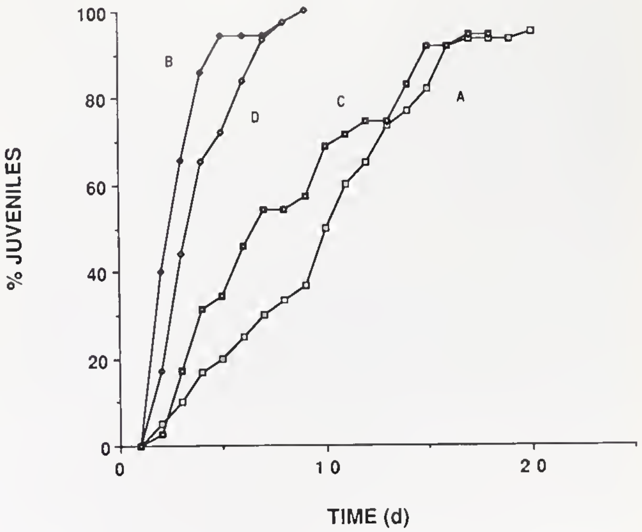

Figure 11. Extracapsular metamorphosis of *Haminoea callidegenita* expressed as the number of individuals that had successfully completed metamorphosis by the indicated day, as a percent of the original number of veligers per substratum. Substrata: (A) seawater only (n = 12 cultures); (B) *Chaetomorpha linum* (n = 7); (C) sediment (n = 7); (D) egg mass jelly (n = 15).

hatched as veligers was enhanced by the presence of *Chaetomorpha* indicating that the factor in *Chaetomorpha* influencing metamorphosis of *H. callidegenita* differs from that of the egg mass jelly.

H. callidegenita veligers were potentially competent to metamorphose over a long period. Some individuals will metamorphose within the egg capsule. The factor(s) which determine which individuals will metamorphose before hatching are unknown, possibly variable sensitivities of the embryos themselves. However, these factors appear to be influenced by egg mass jelly, causing metamorphosis. Other individuals will metamorphose shortly after hatching (1–3 days) if they encounter a favorable substratum, such as a juvenile food source. Still other individuals will delay metamorphosis for 20 days after hatching (potentially longer), until appropriate conditions are met. *H. callidegenita* veligers appear to become increasingly sensitive to metamorphic cues during their development, as veligers hatched from intact egg masses metamorphose in response to jelly pieces, even though jelly before hatching did not provide a strong enough stimulus.

Acknowledgments

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Fine Structural Study of the Cortical Reaction and Formation of the Egg Coats in a Lancelet (= Amphioxus), *Branchiostoma floridae* (Phylum Chordata: Subphylum Cephalochordata = Acrania)

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Abstract. A method for artificial fertilization of lancelet eggs is described, and the egg coats are studied for the first time by transmission electron microscopy. Large, ovarian oocytes and spawned, unfertilized eggs (which are about 140 μm in diameter) are surrounded by a coarsely granular vitelline layer about 1 μm thick and a jelly layer a few micrometers thick. The egg cortex is crowded with a monolayer of cortical granules, each with an average diameter of approximately 3.5 μm. About 20 to 30 s after insemination, a cortical reaction occurs almost simultaneously over the entire egg surface. The cortical granules undergo exocytosis, and part of their content evidently forms a dense layer 30 nm thick against the inside of the vitelline layer: both layers together constitute the fertilization envelope, which begins elevating from the egg surface. By 80 s after insemination, the jelly layer has disappeared, and beneath the fertilization envelope the bulk of the ejected cortical granule material has become organized into a hyaline layer with a finely fibrogranular consistency. By 20 min after insemination, the perivitelline space between the fertilization envelope and the egg surface has attained its maximum width of roughly 150 μm, and both the hyaline layer and the vitelline layer component of the fertilization envelope are much attenuated and remain so until hatching about 9 h after insemination. Egg coats are compared among major deuterostome groups, and the results imply that the ancestral chordate may have been an unspecialized appendicularian.

Introduction

Lancelets, when first discovered in the 1770's, were incorrectly placed in the phylum Mollusca; however, when rediscovered in the 1830's, they were classified without any controversy as primitive fishes (Drach, 1948). In 1865 and 1867, when Kowalevsky described lancelet development, his essential points were that the later embryology and larval development are vertebrate-like, but the early embryology is much like that of an invertebrate deuterostome. These results soon influenced the evolutionary ideas of the time, as discussed by Drach (1948), Dawydoff (1961), and Vucinich (1988). In the mid 1860's, von Baer, one of the world's most respected embryologists, was convinced that there were three groups of invertebrates and one group of vertebrates with no genetic continuity among groups. According to this theory of limited transformationism, there was an unbridgeable gap in the chain of being between the invertebrates and the vertebrates. Kowalevsky's work on lancelet embryology put a quick end to von Baer's notion of limited transformationism and did much to help establish Darwin's conception of a single tree of life. In addition, Kowalevsky ignited a passion for comparative embryology that gripped the German universities until the end of the nineteenth century.

In spite of the impact of Kowalevsky's lancelet work on the history of science, neither he nor the many lancelet biologists who came after him ever established exactly how lancelets are related to the other major groups of animals. Roughly a dozen conflicting schemes for lancelet phylogeny have been proposed, many of which have been reviewed by Haeckel (1893), Drach (1948), Bone (1960), Hennig (1983), and Jefferies (1986). New information should at least help one to discard the most unlikely of these hypotheses, and we have sought to discover useful new character states in the very early development of lancelets.

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Until now, technical problems have forced students of lancelet development to start their descriptions with stages no earlier than the already fertilized egg. Artificial fertilization of lancelet eggs *in vitro* has proven difficult, having previously been accomplished only by Tung *et al.* (1958), who limited their descriptions to blastomere fates and did not concern themselves with earlier development. In other studies of the subject, it has been necessary to initiate lancelet development by allowing males to spawn together with females, either at the time of collection in the field (Kowalevsky, 1865, 1867, 1877; Hatschek, 1882, 1893; Wilson, 1893; Sobotta, 1895, 1897; Van der Stricht, 1896; Cerfonatine, 1906; Conklin, 1932, 1933; Bone, 1958) or in captivity (Lwoff, 1892; Orton, 1913–1915; Webb, 1958; Flood, 1975).

Recently, while studying *Branchiostoma floridae*, a lancelet found along coasts of the southeastern USA, we discovered how to induce spawning in the laboratory. Thus we have been able to investigate the earliest events of development, including sperm-egg interactions, the cortical reaction, the formation of the egg coats, and rearrangements of the egg cytoplasm. The chief purpose of the present paper is to describe the closely related phenomena of the cortical reaction and the formation of the egg coats. The descriptions are based on transmission electron microscopy (TEM) and light microscopy (LM) in conjunction with videotape recording. Surprisingly, this is the first TEM study of any developmental stage of a lancelet younger than the two-week old larva (Flood, 1975). Although our coverage of the first minutes of development is not intended to be comprehensive, we will give context to our work by including a TEM description of the investments of the large oocytes within the ovary and by touching upon polar body emission. The discussion includes a comparison of egg coats between lancelets and other major groups of deuterostome animals.

Materials and Methods

Adult males and females of *Branchiostoma floridae* (Hubbs) (formerly *B. caribaeum*) were collected by shovel and sieve from sandy substratum in water about 1 m deep approximately 100 m south of the Courtney Campbell Causeway in Old Tampa Bay, Florida. After transport to the nearby University of South Florida, the lancelets were distributed individually in dishes of filtered seawater from the field (27‰) at laboratory room temperature (25° ± 1°C). The sexes of the ripe animals were distinguishable because the yellow ovaries or white testes were visible through the body wall. All collections were made during the last week of August and the first week of September, 1988, a time of year when the Tampa Bay population of *B. floridae* was at its ripest according to Nelson (1968); in spite of this, only about 20% of the lancelets in any given collection had gonads of maximum size. Importantly, no males or females spawned while being collected, transported, or maintained in the laboratory—unless we stimulated them as described below.

During the evening of the day of collection, ripe females were stimulated to spawn with a non-lethal electric shock. Each female, lying in a dish of seawater about 2 cm deep, was placed between two platinum electrodes connected to an electronic stimulator (Grass Instrument Co., Quincy, Massachusetts) and shocked for 2 s with 200 direct current pulses each of 10 ms and 50 volts. Since the electric current produced some chlorine electrolytically, the animal was transferred to uncontaminated seawater immediately after shocking. Of about 100 ripe females stimulated, 14 spawned. Approximately 5 min elapsed between stimulation and spawning, which lasted for a few minutes. During each spawning episode, a few hundred to a few thousand eggs were emitted from the atrial pore. Although many of the anterior and posterior gonads were not emptied by spawning, additional electric shocks elicited a second spawning in only two of the females. Once spawned, the eggs remained fertilizable for at least 2 h.

Motile sperm were obtained from the ripe male lancelets by a method so reliable that we did not attempt to induce males to spawn electrically. Because our preliminary results showed that sperm excised from ripe testes were usually immotile in seawater, we induced motility by dissecting the testes in seawater that contained 10 m*M* NH_4Cl and had been adjusted to pH 8 with NaOH. Clouds of motile sperm exiting from the cut testis could be collected by pipette at a concentration roughly equivalent to a 1:500 dilution of dry sperm. Our unpublished TEM showed that this method of inducing motility did not trigger the acrosome reaction.

For insemination, two drops of sperm suspension were added with rapid mixing to approximately 10^3 eggs in 3 ml of filtered seawater. This relatively high concentration of sperm, which fortunately resulted in little polyspermy, helped to ensure simultaneous fertilization of all the eggs. After 30 min, the embryos were transferred to 8 ml of filtered seawater in a petri dish (5 cm in diameter), and thereafter the seawater in the culture dish was renewed at hourly intervals. Depending on the egg batch, a moderate to high proportion of the embryos developed normally, and ciliated neurulae hatched after 9 to 10 h at a temperature of 25° ± 1°C.

TEM fixation, rinsing, and postfixation were by Holland's (1988) glutaraldehyde-dichromate procedure. These solutions were relatively hypertonic to minimize the enormous swelling of the cortical granules that occurred in less concentrated TEM solutions (our unpub. obs.). We fixed large oocytes (contained in fragments of ripe ovaries), spawned eggs, and developmental stages at the following intervals after insemination: 20 s, 40 s, 80 s, 2 min, 4 min, 6 min, 8 min, 10 min, 15 min, 20 min, 40 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, and 8 h. After postfixation, all specimens were dehydrated in an ethanol series and embedded in Spurr's resin. Contrast of silver sections was enhanced with uranyl acetate and lead citrate.

Elevation of the fertilization envelope was recorded on videotape via a video camera (Panasonic model WV3170) attached to a compound microscope. Each egg was inseminated uncompressed in a drop (0.15 ml) of seawater on a microscope slide. From continuous videotape records of six eggs, egg diameters and the elevation of the fertilization envelope were measured every 10 s for 20 min with a stop-frame video cassette recorder (Panasonic model NV8950). All measurements were calibrated with a stage micrometer that had been videotaped with the same apparatus. An additional 37 eggs were measured at 20 min after insemination to determine their diameters and the maximum elevation of their fertilization envelopes.

Results

In Table I, our terminology for the egg investments of *B. floridae* is correlated with that in the earlier literature on lancelet reproduction. In two instances, we created names for structures seen by us for the first time. Otherwise, previously established names have been retained as much as possible. However, since a few of the older terms imply incorrect homologies with structures in other organisms (see discussion), we have substituted more appropriate names.

Investments of large oocytes in the ovary (TEM Fig. 1)

The largest germinal cells within the ovary are primary oocytes, which are generally called ovarian eggs in the older literature. Our TEM has clarified the relationship of the oocyte to the surrounding ovarian tissues, which are the squamous epithelium of the primary ovarian cavity (defined by Zarnik, 1904) and the underlying ovarian blood sinus. The blood sinus is filled with plasma comprising closely packed granules of moderate density, each having a diameter of approximately 15 nm. There is a basal lamina but no endothelium delimiting the ovarian blood sinus, which thus has a fine structure comparable to the rest of the circulatory system in lancelets (Rähr, 1981). When the lancelet oocyte is ovulated into the secondary ovarian cavity (as described by Zarnik, 1904), both the epithelium of the primary ovarian cavity and the blood sinus remain behind as an integral part of the ovarian tissues.

Ovarian oocytes approaching maximal size have a dented surface topography somewhat resembling that of a golf ball. Two extracellular matrices intervene between the oocyte plasma membrane and the blood sinus of the ovary. The matrix closer to the oocyte is the vitelline layer, which is about 0.8 μm thick and consists of dense, oblong granules (about 20 × 60 nm). Short microvilli on the oocyte surface project into the vitelline layer. The more distant matrix is the jelly layer, which is up to 3 μm thick in each surface dent of the oocyte. The jelly consists of loosely packed, fine fibrils, each about 3 nm in diameter. Although our TEM revealed no obvious stages in the synthesis or exocytosis of either matrix by the oocyte, the follicle epithelium, being squamous and located on the outer side of the blood sinus, would not appear to be the source of these matrices. Instead, it is likely that the oocyte itself synthesizes and secretes the jelly layer and the vitelline layer (both of which would thus be primary egg envelopes as defined by Wourms, 1987).

Spawned, unfertilized eggs (TEM Figs. 2–4)

The spawned eggs are, *sensu stricto,* secondary oocytes, having become arrested at the second meiotic metaphase at about the time of ovulation (Van der Stricht, 1896). In *B. floridae,* the living spawned eggs are spherical and their diameters range from 115 to 158 μm, with an average of 138 μm (SD = 9.2; n = 43). The surface dents of the ovarian oocyte have smoothed out, and numerous microvilli about 1 μm long project from the surface of the spawned egg and penetrate most of the thickness of the vitelline layer, which is now 1.2 μm thick. The jelly layer, which is at most about 1.5 μm thick in TEM preparations, is located just outside the vitelline layer. The jelly forms a continuous layer around some eggs, but is more patchily distributed on the surface of others. Evidently the jelly layer is not much thicker in the living state, because no obvious spaces separate living eggs crowded in a monolayer and observed by LM.

The cortex of the unfertilized egg is dominated by a monolayer of cortical granules each of which is ovoid to pyriform (averaging 2.5 μm wide × 4.5 μm long) with its long axis perpendicular to the overlying plasma membrane (Fig. 2). The bounding membrane of the granule is usually broken in many places in our TEM preparations due to some swelling of the contents, which consist of a packed mass of 30 nm granular or fibrillar material of relatively low electron density. Most of the cortical granules are separated from the overlying plasma membrane by no more than about 50 nm of intervening cytoplasm. The cytoplasmic regions separating adjacent cortical granules in the egg cortex contain abundant free ribosomes, some profiles of endoplasmic reticulum and a very few mitochondria. This cortical cytoplasm (Fig. 3) typically appears denser than the deeper cytoplasm (Fig. 4) in our electron micrographs, possibly due to the un-

Table I

Investments of lancelet eggs: comparison of terms used in the present and previous studies (Italian, German, and French terms have been translated literally into English)

LARGE OOCYTE IN OVARY

Present study (TEM)	Zarnik, 1904 (LM)	Reverberi [illegible], 1971 (TEM)	Reverberi and De Leo, 1972 (TEM)	Guraya, 1978, 1983 (LM)
Epithelium of primary ovarian cavity	Follicle epithelium	External follicle	Cellular envelope	Thecal layer
Blood sinus	Blood sinus	Internal follicle	Gelatinous substance	Basal lamina
Jelly layer	No mentioned	Not mentioned	Not mentioned	Not mentioned
Vitelline layer	Not mentioned	Niche material	Granular sheet	Zona pellucida

UNFERTILIZED EGG

Present study (TEM)	Sobotta, 1897 (LM)	Cerfontaine, 1906 (LM)
Jelly layer	Not mentioned	Not mentioned
Vitelline layer	External membrane	Vitelline membrane

FERTILIZED EGG

Present study (TEM)	Sobotta, 1897 (LM)	Several 19th century references (LM)[1]	Cerfontaine, 1906 (LM)	Several 20th century references (LM)[2]	Recent reviews[3]
Jelly layer	Not mentioned	Not mentioned	Not mentioned	Not mentioned	Not mentioned
Fertilization envelope	External membrane	Vitelline membrane	Vitelline membrane	Fertilization membrane	Fertilization membrane
Hyaline layer	Internal membrane (= Chief membrane)	Not mentioned	Perivitelline membrane	Not mentioned	Jelly-like substance

[1] Kowalevsky, 1867; Hatschek, 1882; Willey, 1894; Van der Stricht, 1896.

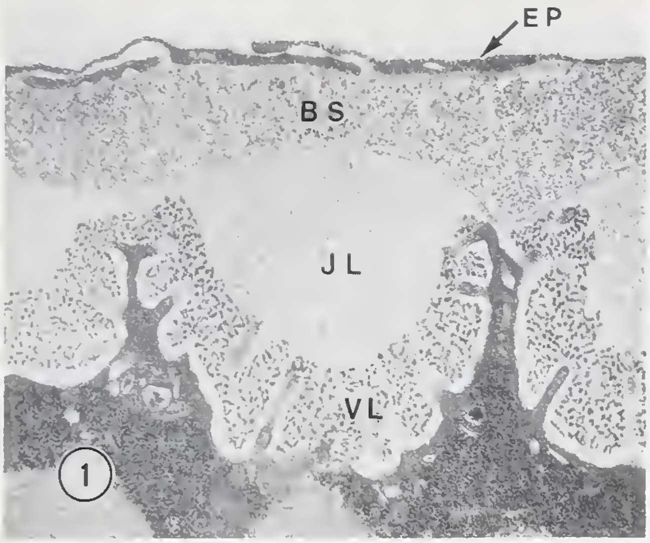
[2] Conklin, 1932; Tung *et al.*, 1958; Wickstead, 1973.

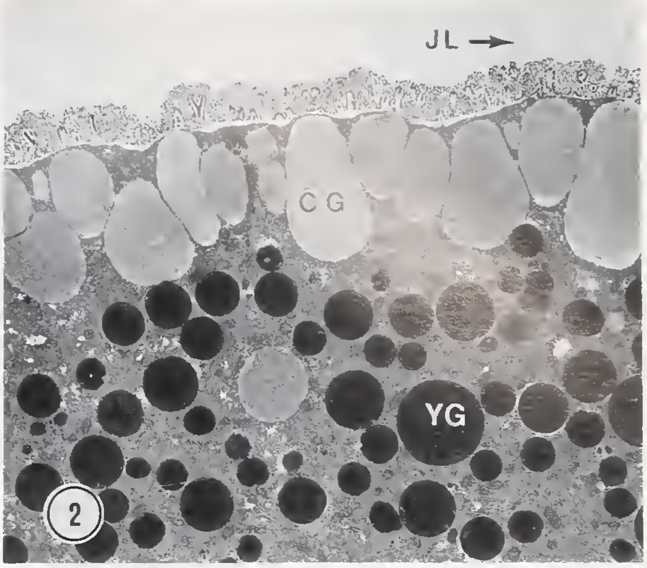
[3] Reverberi, 1971; Guraya, 1983.

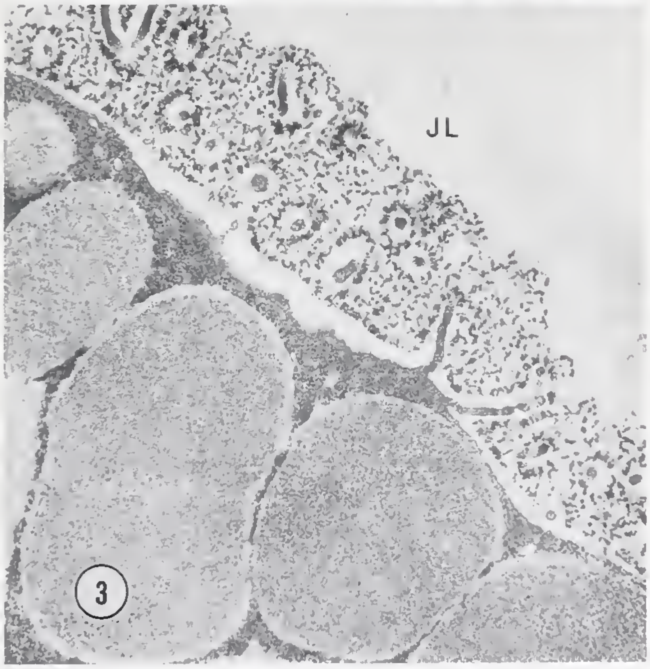
avoidable swelling of the cortical granules during TEM processing. The cytoplasm beneath the cortical zone includes numerous ribosomes, many mitochondria and yolk granules. The yolk granules, which range in diameter from about 2 to 5 μm, have a complicated structure including fibrillar components that Song and Wu (1986) have previously described by TEM for the yolk granules of ovarian oocytes in *B. belcheri*. The TEM of the remaining cytoplasmic and chromosomal structures is beyond the scope of the present study.

Elevation of the fertilization envelope (LM, Figs. 5–8)

About 20 to 30 s after insemination, the elevation of the fertilization envelope begins (the TEM of this structure will be described in the next section of the results), and a perivitelline space opens up between the envelope and the plasma membrane of the egg. In some eggs, elevation begins a few seconds earlier at the animal pole (conveniently marked by the presence of the first polar body) than elsewhere on the egg surface; however, in many eggs, elevation begins simultaneously over the entire surface. Continuous videotape records of six eggs (Fig. 5) show that the rate of elevation was greatest in the first few minutes after insemination and that the first polar body adheres to the outside of the elevating fertilization envelope as the perivitelline space widens (Figs. 6–8). The videotapes also demonstrate that egg diameter may sometimes shrink slightly (at most by 5 μm) as elevation of the fertilization envelope begins; however, such shrinkage does not always take place. In the first few minutes after insemination, eggs remain nearly spherical and undergo deformations that are at most slight (compare Figs. 6 and 7) if detectable at all. In the lancelet, fertilization is not followed by conspicuous deformations comparable to those described for some ascidian eggs (Sawada, 1988). In the eggs of *B. floridae*, the most marked change in egg shape is a little flattening at the animal pole during the emission of the second polar body approximately 8 min after insemination. In the present study, we made no observations on living eggs before spawning; therefore, it remains possible that there are additional, very early episodes of egg deformation—either before or during first polar body emission.







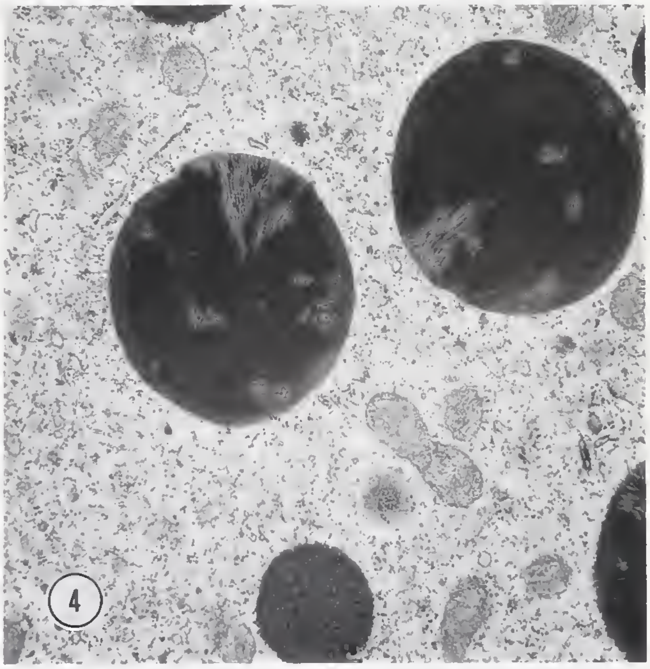


Figure 1. TEM of the ripe ovary of a lancelet. The periphery of a large oocyte (at bottom with pitted surface topography) is surrounded by a vitelline layer (VL) and, external to that, a jelly layer (JL). The associated ovarian tissues are the blood sinus (BS) and the epithelium lining the primary ovarian cavity (EP), which are left behind in the ovary when the oocyte along with its vitelline layer and jelly layer are ovulated. ×18,000.

Figure 2. TEM of a lancelet egg that has been spawned but not fertilized. The jelly layer (JL) is patchily distributed and adheres to the outside of the vitelline layer. Most of the egg cortex is occupied by a monolayer of cortical granules (CG), and yolk granules (YG) are the most conspicuous organelles deeper in the cytoplasm. ×3000.

Figure 3. TEM of the periphery of a lancelet egg that has been spawned but not fertilized. The jelly layer (JL) overlies the vitelline layer, and cortical granules are the predominant organelle in the egg cortex (toward the lower left). ×18,000.

Figure 4. TEM of the deeper cytoplasm of a lancelet egg that has been spawned but not fertilized. There are numerous free ribosomes, mitochondria, and yolk granules. Near the top left, there is a profile of rough endoplasmic reticulum. ×18,000.

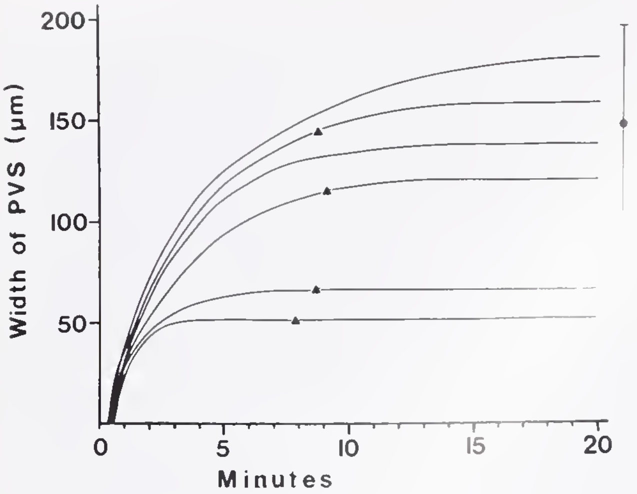


Figure 5. The time course of the elevation of the lancelet fertilization envelope. For six eggs, the width of the perivitelline space (PVS) is plotted against time in minutes after insemination. The data were gathered from video recordings made through the LM. For four of the eggs, triangles mark the time of second polar body emission (the other two eggs were unfavorably oriented and the second polar body was not visible). The filled circle shows the mean width of the perivitelline space of 43 eggs measured 20 min after insemination; the error bars are ±1 standard deviation.

For most eggs, the maximum width of the perivitelline space (147 µm; SD = 46; n = 43) is reached by 20 min after insemination. In spite of considerable scatter in the data, there is a significant (P = 0.008) correlation between egg diameter and width of the perivitelline space 20 min after insemination (Pearson product moment statistic = 0.401; r^2 = 0.141). The regression is: space width = egg diameter × 2.03 − 128.7. The mean width of the perivitelline space just before hatching (9 h after insemination) is 165 µm (SD = 24; n = 10). Due to the large standard deviations, there is no significant difference between the mean widths at 20 min and 9 h.

Cortical reaction 40 s after insemination (TEM Figs. 9–10)

In *B. floridae* the exocytosis of the cortical granules takes place over the entire egg surface almost simultaneously; there is no definite progression of the cortical reaction in a wave sweeping over the egg surface such as in echinoids. Scattered cortical granules may briefly lag behind their neighbors in undergoing exocytosis, but within a few seconds the egg cytoplasm contains virtually no unreacted cortical granules. At exocytosis, the cortical granule membrane fuses with the overlying plasma membrane, and the granule contents swell and appear as a mass of loosely packed granular and fibrillar material. As the cortical granule material pushes the vitelline layer away from the egg surface, the vitelline layer becomes thinner (reduced to only 0.3 µm in some places). Moreover, another layer (Fig. 10, arrows), previously undescribed, forms where cortical granule material comes into contact with the inside of the vitelline layer. We will call this new layer, which is about 20 nm thick and has a dense, very finely granular consistency, the *inner layer of the fertilization envelope:* from this time in development, we will call the vitelline layer the *outer layer of the fertilization envelope.* It is likely that the inner layer forms from cortical granule material, although this point cannot be proven unequivocally from static morphological data. The thin jelly layer is still present on the surface of the fertilization envelope.

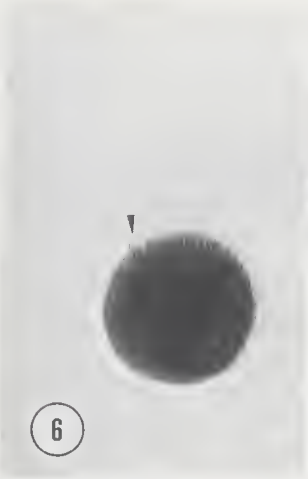
Completion of the hyaline layer 80 s after insemination (TEM Figs. 11–12)

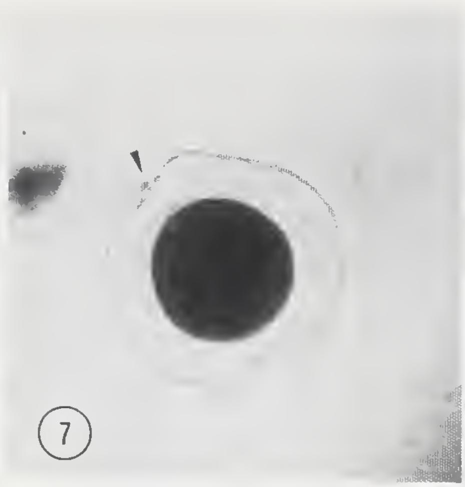
At 80 s after insemination, most of the cortical granule material expelled into the perivitelline space has become confluent to form a hyaline layer, which was first described from LM sections by Sobotta in 1897 (see Table 1). This layer is conspicuous in TEM, although almost invisible in LM of living material. The recently formed hyaline layer has an average thickness of roughly 10 µm and is composed of a diffuse, finely fibrogranular material with a low electron density. Scattered within the hyaline layer, there are some membranous profiles that are probably the remains of microvilli torn away from the egg surface during the cortical reaction. The space between the inner surface of the hyaline layer and egg plasma membrane also contains abundant microvilli, at least some of which are still part of the egg surface. Peripheral to the hyaline layer, the fertilization envelope consists of a 200 nm thick outer layer (derived from the vitelline layer) and a 30 nm thick inner layer (evidently derived from cortical granule material). There is no longer any trace of the jelly layer that was formerly located on the surface of the fertilization envelope.

Fertilization envelope and hyaline layer up to hatching (TEM Figs. 13–14)

During the first few minutes after insemination, not only the fertilization envelope, but also the hyaline layer elevates from the plasma membrane of the fertilized egg. In not remaining closely associated with the egg surface, the hyaline layer resembles that of ophiuroids (Holland, 1979) and not that of echinoids (Chandler and Heuser, 1980; Cameron and Holland, 1985).

Both the hyaline layer and the outer layer of the fertilization envelope rapidly become thinner as the perivitelline space widens. By 20 min after insemination, the hyaline layer has a width of 1 µm or less, and the outer layer of the fertilization envelope is reduced to about 25 nm





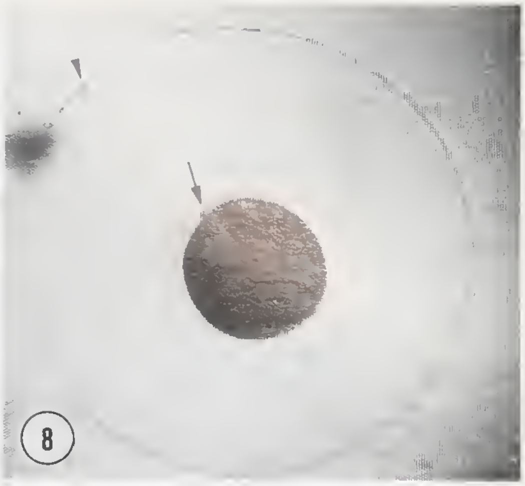


Figure 6. Videotape LM of a lancelet egg that has been spawned but not fertilized; the arrowhead marks the first polar body. ×140.

Figure 7. Videotape LM of a lancelet egg 80 s after insemination. The first polar body (arrowhead) adheres to the outside of the rising fertilization envelope. ×140.

Figure 8. Videotape LM of a lancelet egg 20 min after insemination. The first polar body (arrowhead) adheres to the fully risen fertilization envelope, and the second polar body (arrow) adheres to the animal pole of the egg. ×140.

and may be interrupted in some places. It is not clear whether such interruptions are due to abrasion or extreme stretching (by 20 min after insemination, the surface area of the risen fertilization envelope is about ten times its original surface area). In contrast, the inner layer of the fertilization envelope is complete and, if anything, even thicker (45 nm) than it was previously. It is possible that part of the substance of the hyaline layer is continuously added to the fertilization envelope's inner layer to maintain the integrity of the latter. As the time of hatching approaches (about 9 h after insemination), the thickness of the hyaline layer has been reduced to about 150 nm, due perhaps to the influence of the embryo's ciliated epidermis or to a hatching enzyme secreted by the embryo. Moreover, the outer layer of the fertilization envelope is completely absent in many places. Apparently, the chief barrier that the embryo must pass through at hatching is the inner layer of the fertilization envelope.

Discussion

Possible functions of lancelet egg coats

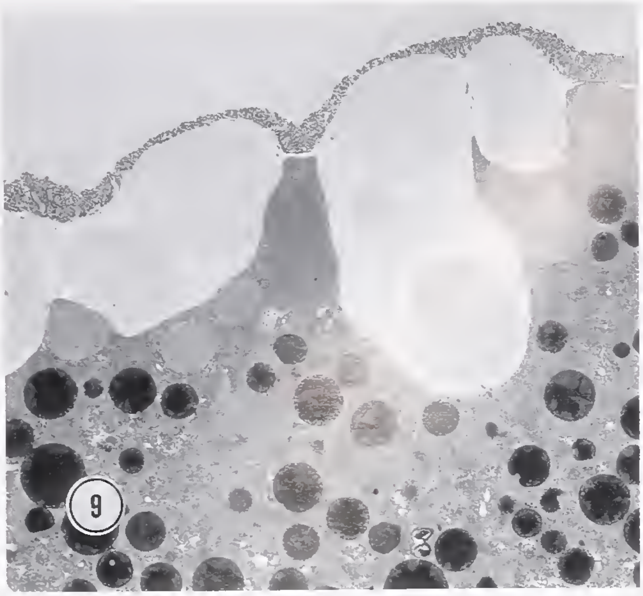
The jelly layer and/or the vitelline layer of the unfertilized lancelet egg probably interact with approaching sperm to trigger the acrosome reaction and then serve as sites for the initial binding of the sperm. Sobotta (1897) shows sperm bound to the elevating fertilization envelope of a lancelet in his LM illustrations, but a thorough TEM study of sperm binding and entry is needed.

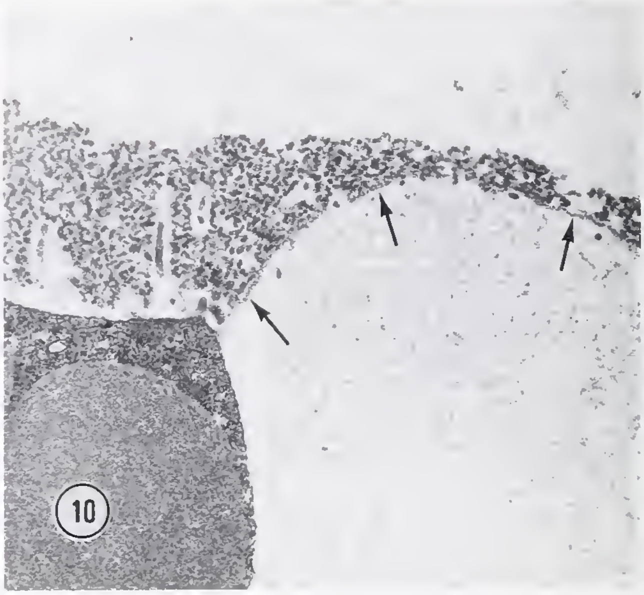
Although nothing definite is known about polyspermy blocks in lancelets, it may well be that the formation of the fertilization envelope establishes a slow, permanent block following a fast, transient block due to a fertilization potential at the level of the plasma membrane. Such a dual system to ensure monospermic fertilization is well known in some other deuterostomes (reviewed in Mazzini *et al.*, 1984, and Elinson, 1986).

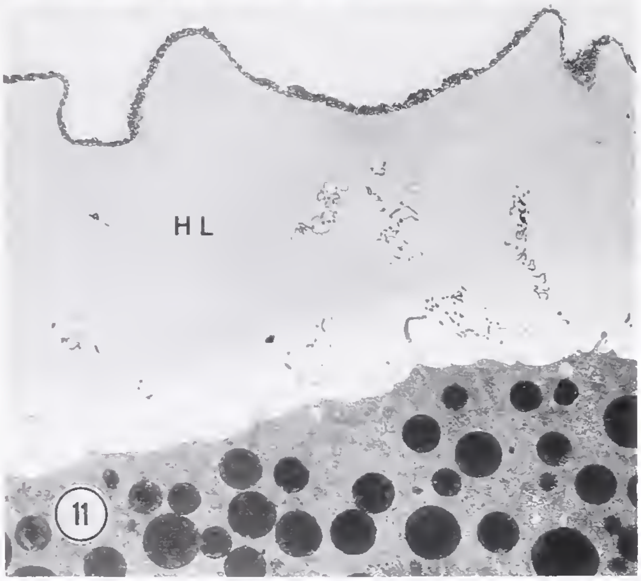
Removal of both the fertilization envelope and the hyaline layer has no adverse effect on lancelet development (Tung *et al.*, 1958), and thus these egg coats are not required for normal morphogenetic movements (*e.g.*, gastrulation) in laboratory culture. In the natural environment, however, the egg coats probably play a role in protecting the developing embryos from mechanical damage, microbial attack, and predation. Our incidental observations on living and fixed material have shown that eggs and embryos within an elevated fertilization envelope sink much more slowly than unfertilized eggs. This phenomenon, which deserves more careful study, should have important consequences for the bathymetric distribution and lateral transport of lancelet embryos in nature.

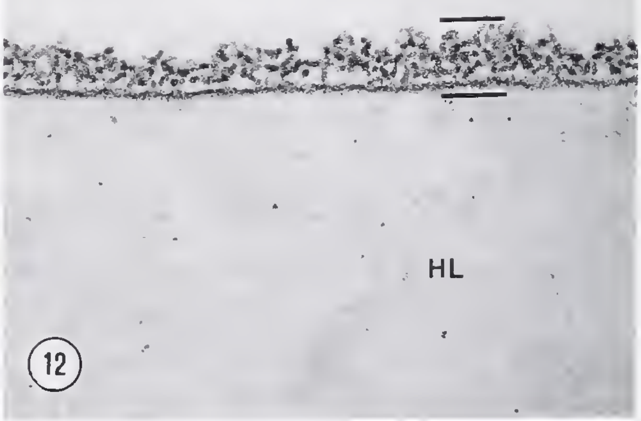
Comparison of egg coats among deuterostomes

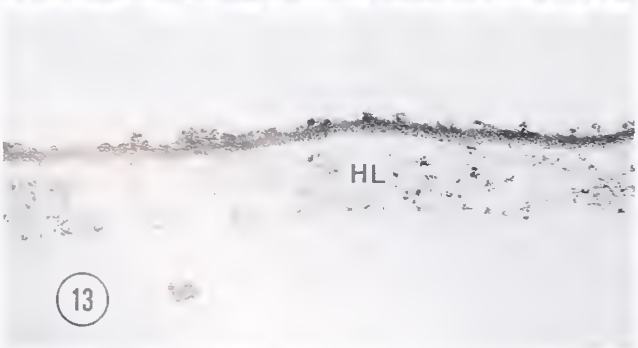
We will limit this discussion to deuterostomes in a restricted sense (*i.e.*, as comprising the echinoderms, hemichordates, and chordates, but excluding the enigmatic chaetognaths). Readers wishing a review of egg coat evo-











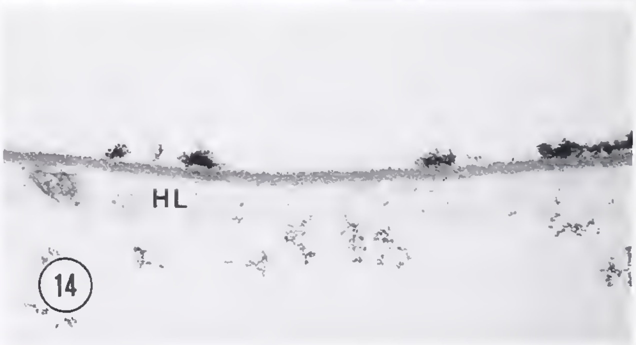


Figure 9. TEM of a lancelet egg 40 s after insemination while the cortical reaction is in progress. Important features from top to bottom are: the inconspicuous jelly layer, the vitelline layer, ejected contents of cortical granules in the perivitelline space, and the egg cytoplasm still containing some unreacted cortical granules and numerous yolk granules. ×3000.

Figure 10. TEM of the periphery of a lancelet egg 40 s after insemination. The inconspicuous jelly layer (at top) overlies the vitelline layer, which is being converted into the fertilization envelope by the

lution for the entire animal kingdom are directed to Mazzini *et al.* (1984). The present discussion will largely exclude homologies among the ovarian layers, because their embryonic origins and fine structures are in need of further study in some lower deuterostomes: thus we will concern ourselves chiefly with the coats surrounding the spawned egg before and after fertilization. These egg coats usually have little structural complexity even as seen by TEM, and, when such uncomplicated structures are compared, homoplasies are most easily mistaken for homologies (Ruppert, 1982). Therefore, the homologies connoted by our terminology should remain tentative until additional information (especially on the detailed biochemistry) is at hand. Unless explicitly stated below, the references are for species that spawn their eggs freely into the water.

Hemichordates and echinoderms compared to lancelets. Before fertilization, the coats of the spawned lancelet egg have most in common with hemichordate and echinoderm eggs. The coat just outside the egg plasma membrane is the vitelline layer, and the coat just outside the vitelline layer is the jelly layer in enteropneust hemichordates (Hadfield, 1975), in crinoids (Holland, 1977) in ophiuroids (Holland, 1979; Yamashita, 1984), in echinoids (numerous references, the most comprehensive being Chandler and Heuser, 1980), and in asteroids (Holland, 1980; Longo *et al.*, 1982; Souza and Azevedo, 1988). In comparison to echinoderms, lancelets have a much thicker vitelline envelope with a more coarsely granular structure. The initial thickness of the layer in lancelets may be related to the great attenuation it must undergo during the enormous expansion of the perivitelline space after fertilization.

As in lancelets, the fertilized eggs of the hemichordates and echinoderms mentioned in the previous paragraph undergo cortical granule exocytosis, and some of the cortical granule contents become added to the vitelline layer to produce a composite structure, the fertilization envelope. The hyaline layer, which forms in the perivitelline space from other components of the cortical granules, has been described only for the lancelet and for some echinoderms (namely, echinoids, ophiuroids, and, more arguably, asteroids). However, for enteropneust hemichordates, existing TEM studies are not adequate to establish whether a hyaline layer is present, and further work is needed.

The unfertilized eggs of some hemichordates and a few echinoderms may lack egg coats present in unfertilized lancelet eggs. Pterobranch hemichordates appear to have no egg coats of any kind (Lester, 1988), perhaps because they brood their eggs and embryos, and no vitelline layer can be demonstrated in holothurians (Holland, 1981).

Tunicates (phylum Chordata: subphylum Urochordata) compared to lancelets. Among the tunicates, appendicularians have egg coats most similar to lancelet egg coats (Holland *et al.*, 1988). The unfertilized appendicularian egg is surrounded by a vitelline layer, but lacks a jelly layer. There are no follicle cells outside the vitelline layer in several species of appendicularians, and never any test cells beneath it. After fertilization, appendicularian eggs undergo cortical granule exocytosis, and some of the contents of these organelles ultimately adhere to the vitelline layer. However, no trace of a hyaline layer ever forms.

Other tunicates, whether they brood their eggs or spawn them freely in the water, have some of the most unusual egg coats in the animal kingdom. The vitelline layer (often called the chorion in the ascidian literature) is underlain by scattered test cells and overlain by a layer of follicle cells (Berrill, 1975; Lambert and Koch, 1988). Moreover, the egg has no jelly layer. After fertilization, there are no cortical granules in the egg to undergo exocytosis, and the egg coats, as seen by TEM, remain virtually unchanged. Therefore, the egg coats of tunicates

addition of a thin inner layer (arrows) to its inner surface. This inner layer of the fertilization envelope is probably derived from part of the ejected cortical granule contents. An unreacted cortical granule remains in the cortical cytoplasm at the lower left. ×18,000.

Figure 11. TEM of a lancelet egg 80 s after insemination. The jelly layer has disappeared, and the fertilization envelope (at top) is elevating from the surface of the egg (at bottom). Much of the intervening perivitelline space is occupied by the hyaline layer (HL) formed from the ejected contents of the cortical granules. The membranous profiles in the perivitelline space are probably microvilli torn away from the egg surface during the cortical reaction. ×3000.

Figure 12. TEM of the lancelet fertilization envelope (between the two parallel lines) 80 s after insemination. The thicker outer layer is derived from the former vitelline layer, and the thin inner layer is evidently derived from cortical granule material. The outer part of the hyaline layer (HL) appears in the bottom half of the figure. ×30,000.

Figure 13. TEM of the lancelet fertilization envelope 20 min after insemination. The outer layer of the fertilization envelope and the hyaline layer (HL) are both much thinner than before. ×30,000.

Figure 14. TEM of the lancelet fertilization envelope 8 h after insemination (1 h before hatching). Only scattered fragments of the outer layer of the fertilization envelope remain, and the hyaline layer (HL) is relatively inconspicuous. ×30,000.

other than appendicularians are very different from those of lancelets. Unfortunately, Reverberi (1966, 1971) had a strongly preconceived notion that lancelet eggs should resemble ascidian eggs, and he was led into making some egregiously incorrect homologies, as Guraya (1978, 1983) has justly pointed out. Reverberi, in the first TEM studies of lancelet oocytes in the ovary, identified the vitelline layer as the equivalent of the test cells in ascidians, and he identified the ovarian blood sinus as a layer of follicle cells: in truth, neither the vitelline layer nor the blood sinus of lancelets is a cellular structure at any time during oogenesis. In 1972, Reverberi and De Leo gave the structures in question less definite names (Table I), but by then the original misinterpretations were well entrenched in the literature.

Lower vertebrates (phylum Chordata: subphylum Vertebrata) compared to lancelets. Guraya (1978, 1983), in LM studies of lancelet ovaries, believed that the entire blood sinus (that is the blood plasma and its bounding basal laminae) was a single, very thick basal lamina. To Guraya, such a basal lamina appears homologous to the one separating the follicle from the theca in vertebrate ovaries, and his names for the other investments of the oocytes (Table I) are also taken from vertebrate ovarian histology. In short, he considers the ovarian oocyte of a lancelet to be like that of a vertebrate without its follicle cell layer. We partially agree with Guraya's implied homology between the vertebrate zona pellucida and the lancelet vitelline layer. However, it is likely that the lancelet vitelline layer is a primary envelope, while the zona pellucida of lower vertebrates (cyclostomes and fishes) is a compound envelope (*sensu* Wourms, 1987), having been produced by both the oocyte and the follicle cells (Hardisty, 1971; Laale, 1980; Dumont and Brummett, 1985). Any jelly coating the outside of the zona pellucida of vertebrate eggs is secreted by the oviduct and is thus not homologous to the jelly layer of lancelets, hemichordates, and echinoderms.

Fertilization in the vertebrate egg often results in cortical granule discharge (although not in elasmobranchs, urodeles or birds, which lack such organelles). A few TEM studies of fertilized eggs of vertebrates have indicated that cortical granule material is added to the zona pellucida (= vitelline layer); *i.e.*, in anurans the added material evidently forms the F layer (Grey *et al.*, 1974). In most vertebrates, however, such additions are inconspicuous if detectable at all by TEM (Dumont and Brummett, 1985). Finally, structures comparable to the hyaline layer of lancelets and some echinoderms have almost never been found in the perivitelline space around any vertebrate egg: the only possible hyaline layer homologue described to date for any vertebrate is the S layer surrounding the fertilized anuran egg (Larabell and Chandler, 1988a, b).

Implications of the present study for lancelet phylogeny

The new information in the present paper is not extensive enough to warrant a full review of the many proposed schemes of lancelet phylogeny. Even so, two of these schemes can profitably be re-examined in the light of our results. At the outset, we should state that we think the hemichordates and echinoderms are appropriate outgroups for analyzing relationships within the chordates.

We will consider phylogenetic scenarios deriving lancelets from tunicate-like antecedants, since we favor the commonest cladistic arrangement of the chordate subphyla: namely that the tunicates are the sister group of the lancelets plus vertebrates (Hennig, 1983; Ghiselin *et al.*, 1986). By outgroup comparison, all the character states of the lancelet egg coats are plesiomorphic for the phylum Chordata. This means that any group interposed between the lancelets and the base of the phylum Chordata should have egg coats with as few apomorphies as possible.

The appendicularian tunicates have fewer apomorphies (the loss of the jelly layer and the loss of the hyaline layer) than the ascidian and thaliacian tunicates, which have the following apomorphies: loss of the jelly layer, loss of the hyaline layer, loss of cortical granules, addition of test cells, and addition of follicle cells. If one assumes that the stem tunicate was an appendicularian-like animal with eggs like modern appendicularians, then lancelets derived from it need only regain the jelly layer and hyaline layer. The alternative that eggs of the appendicularian-like stem tunicate had not yet lost the jelly and hyaline layers, would mean that lancelet eggs would not have had to regain any structures. In contrast, if one assumes that the stem tunicate was an ascidian-like animal with eggs like those of modern ascidians, then lancelets derived from it must lose test cells and follicle cells while regaining the cortical granules, jelly layer and hyaline layer. Thus, from a consideration of egg coats in deuterostomes, we suggest that the ancestral chordate resembled an unspecialized appendicularian (a theory best developed by Brooks in 1893) and was not like an adult ascidian that developed a tadpole larva which became neotenous and gave rise to lancelets and vertebrates (a theory best developed by Garstang in 1928).

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An Ultrastructural Investigation of Spermatogenesis in the Holopelagic Polychaetes *Vanadis formosa* and *Krohnia lepidota* (Polychaeta: Alciopidae)

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Abstract. The earliest stages encountered were clusters of spermatogonial cells floating free in the coelom. Neither germinal epithelium nor definitive gonad was observed. Mitotic divisions of spermatogonial cells resulted in large coelomic clusters containing up to 800 cells each. Cells within these clusters were connected to neighboring cells via cytoplasmic bridges that were characterized by a dense collar around the cell membrane and by microtubules traversing the common cytoplasm between cells. Peripheral cells detached from large cell clusters and entered meiotic divisions in the coelom. Spermatid nuclei initially contained granular chromatin that became fibrillar and eventually electron opaque as nuclear elongation and condensation proceeded. The acrosome first appeared in the posterior cytoplasm as a proacrosomal vesicle with an associated Golgi complex. The plate-like proacrosome migrated toward the anterior end of the sperm and reached the apex prior to completion of nuclear condensation. The middlepiece contained 5–7 mitochondria in *Vanadis formosa* and 8–10 mitochondria in *Krohnia lepidota*. Two centrioles, perpendicular to each other, were located in the middlepiece with the proximal centriole residing in a shallow fossa at the base of the nucleus. The distal centriole was associated with a nine-spoke, branched anchoring apparatus at the posterior edge of the middlepiece. Sperm development and mature sperm ultrastructure were very similar in *V. formosa* and *K. lepidota*. The mature coelomic sperm of *K. lepidota* were longer than those of *V. formosa* (8.7 μm *vs.* 11.0 μm, nucleus + middlepiece). The posterior nuclear region of *V. formosa* sperm was irregular in shape while that of *K. lepidota* was symmetrical. A series of dense accessory "membranes" was observed between the nucleus and the plasmalemma of *K. lepidota* sperm but not in *V. formosa*. Stored sperm in female worms were observed in *V. formosa* but no female *K. lepidota* were available in this study. Sperm storage and elongated sperm structure in alciopid polychaetes may be related to the pelagic environment in which they live and the suspected infrequent encounters between individuals.

Introduction

The Alciopidae is one of six holopelagic polychaete families whose representatives spend their entire life history in the water column (Dales and Peter, 1972). Alciopids have evolved a number of specialized features for pelagic existence including two large, highly developed eyes, long thin transparent bodies, and specialized reproductive characteristics. The structure and function of the complex eyes of alciopids have been described by Hermans and Eakin (1974) and Wald and Rayport (1977). The fragility of alciopids and the problems associated with collection of healthy, intact individuals have precluded detailed ultrastructural studies of gametogenesis until recently. Rice (1980, 1984, 1987) has reported on the *in situ* behavior, reproductive biology, and evolution of 14 alciopid species from the western Atlantic Ocean. Eckelbarger and Rice (1988) have described the ultrastructural events of oogenesis in two species of alciopids.

Alciopid polychaetes are believed to have arisen from ancestral stock similar to the present-day phyllodocid,

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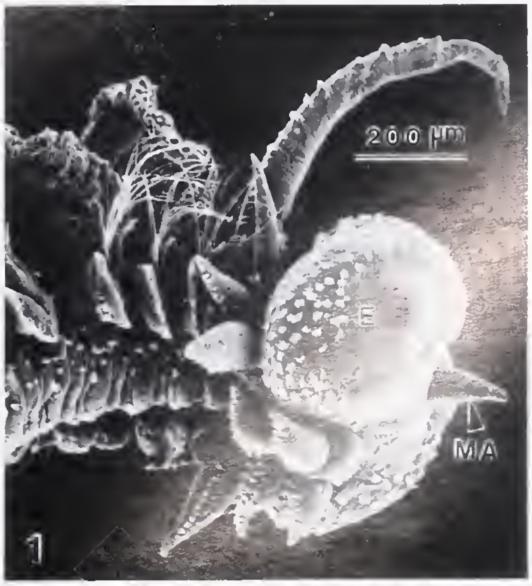
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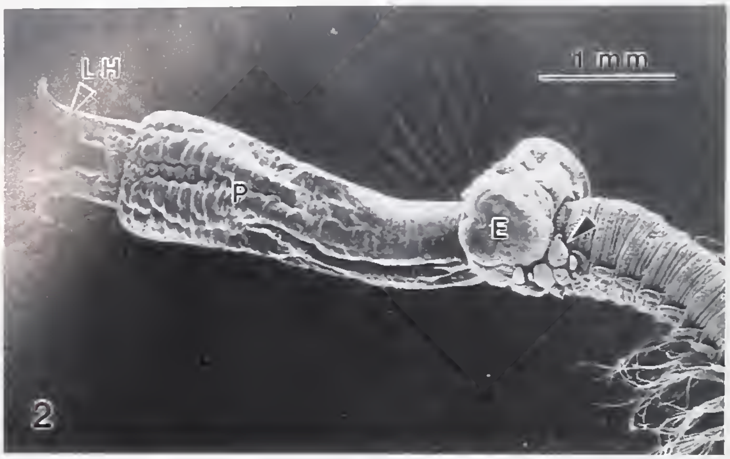
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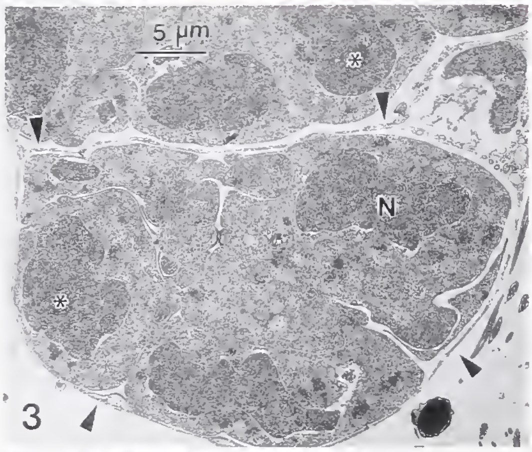
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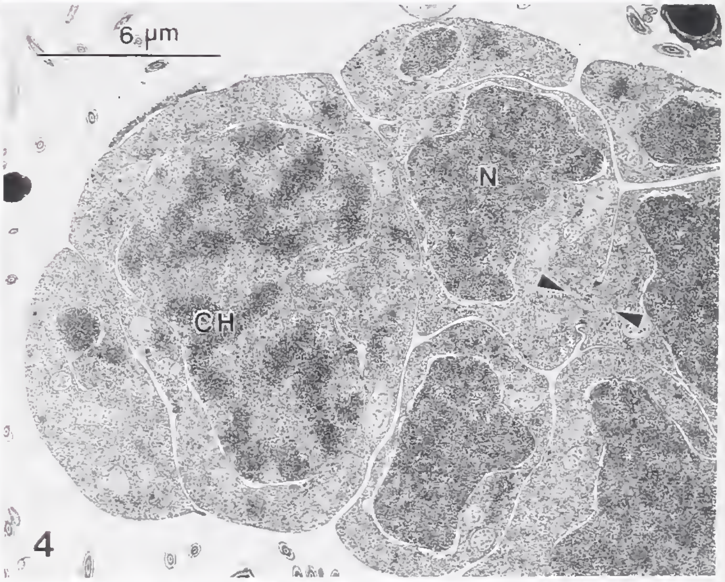
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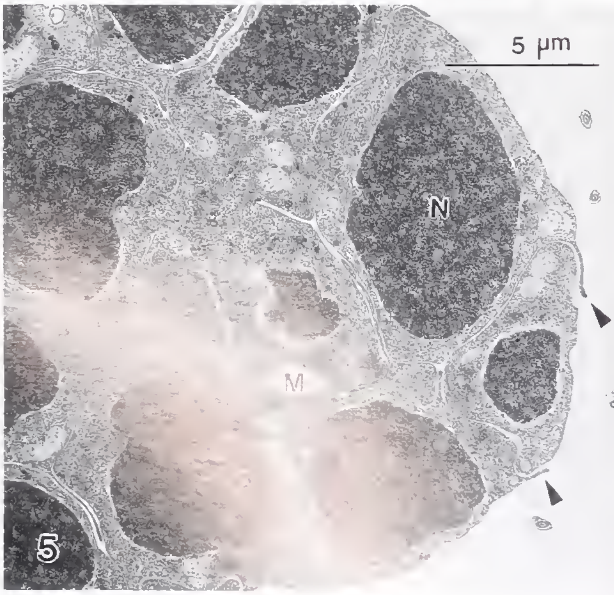
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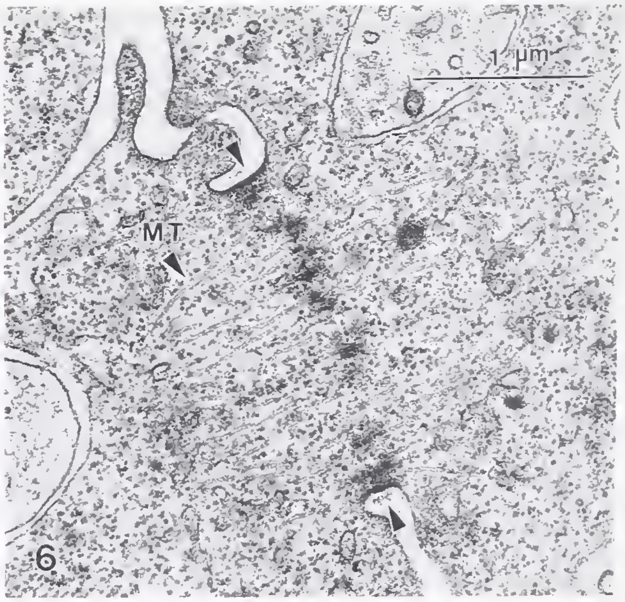
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Eulalia (Dales, 1955; Ushakov, 1972). Detailed ultrastructural information on alciopids will be central to testing this hypothesis of evolutionary descent of the family and also for establishing generic relationships within the Alciopidae.

Spermatogenesis has not been previously described in any species of Alciopidae. Rice (1987) presented information on the external morphology of mature sperm from eight genera and indicated that considerable diversity exists between sperm from different genera within the family. Franzén and Rice (1988) presented micrographs of the mature sperm of *Naiades cantrainii, Torrea candida,* and *Krohnia lepidota* in their review of polychaete sperm ultrastructure but did not describe these sperm or their development in detail. Studies of sperm morphology and sperm development have proven useful in other polychaetes in analyzing systematic relationships and phylogenetic position (Rice, 1981; Pfannensteil *et al.,* 1987; Eckelbarger and Grassle, 1987). The Alciopidae has been poorly studied compared to other polychaete families and the present study was intended to provide new information that may have implications for the generic relations within the family as well as the relationship between the Alciopidae and other polychaete families. The variety of reproductive adaptations seen in the Alciopidae suggests a possible evolutionary scheme that may be shared by other pelagic animals.

Materials and Methods

The animals in this study were collected (by S.A.R.) from the Northwest Providence Channel, Bahama Islands, using a JOHNSON-SEA-LINK submersible operated by Harbor Branch Oceanographic Institution. Collections were made in September and November, 1979, and in April, 1980, with both *Krohnia lepidota* (Krohn, 1845) and *Vanadis formosa* Claparede, 1870, collected on each occasion. *Vanadis formosa* (Fig. 2) was the most abundant species encountered of the 14 species collected (Rice, 1987), with a total of 59 specimens collected at depths of 80–180 m. *Krohnia lepidota* (Fig. 1) was encountered only occasionally with a total of 12 specimens collected at depths of 90–150 m.

The majority of *Vanadis formosa* collected were males with an overall sex ratio of 6:1. All specimens of *Krohnia lepidota* collected were males, although this probably reflects unavoidable sampling error due to the limitations of the collecting techniques and the small number of individuals collected.

Living animals were captured and returned to the surface using the specialized zooplankton collection device on the JOHNSON-SEA-LINK. Specimens were fixed within one hour of collection for scanning (SEM) and transmission (TEM) electron microscopy. Specimens for TEM were fixed for two hours at room temperature (23–25°C) in 2.5% glutaraldehyde buffered with 0.2 *M* sodium phosphate followed by postfixation for 1 h in 1% osmium tetroxide buffered with 1.25% sodium bicarbonate. Specimens were identified following postfixation, then dehydrated in increasing concentrations of ethanol, transferred through two changes of propylene oxide, and embedded in Epon. Sections were cut on a Porter-Blum MT-2B ultramicrotome with a diamond knife, stained with aqueous saturated uranyl acetate and lead citrate, and examined with a Zeiss EM9-S2 transmission electron microscope. SEM specimens were fixed as above followed by dehydration to amyl acetate. Critical point drying was accomplished using liquid CO_2 as the transition solvent. Dried specimens were mounted and coated with gold-palladium and viewed with a Zeiss Novascan 30 scanning electron microscope.

Results

Spermatogenesis

Spermatogenesis in *V. formosa* and *K. lepidota* occurs in the spacious coelomic cavity of the animal. No gonad was found in either species and no conclusive ultrastructural evidence confirming the sequence of mitotic and meiotic divisions was encountered. It is proposed that clusters of spermatogonial cells arise from the coelomic

Figure 1. Lateral view of head of *Krohnia lepidota* viewed with SEM. E, eye; MA, median antenna.

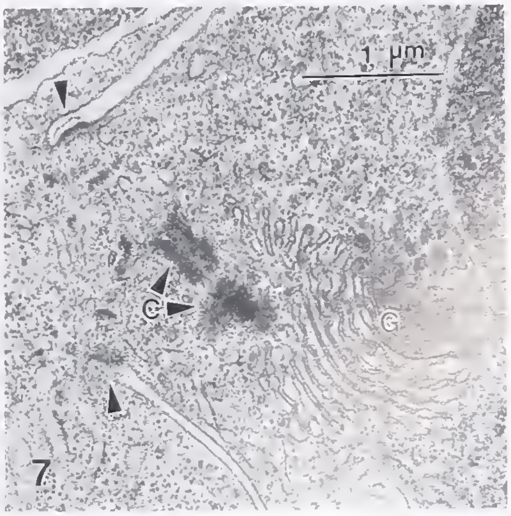
Figure 2. Lateral view of head of adult female *Vanadis formosa* viewed with SEM. E, eye; P, extended proboscis; LH, lateral horn of proboscis. Arrow indicates seminal receptacle.

Figure 3. Clusters of spermatogonia with irregular nuclei (N) and small nucleoli (*) in *V. formosa.* Note thin layer of sheath cells surrounding cell clump (arrows).

Figure 4. Cluster of spermatocytes showing irregular nuclei (N) and intercellular bridge (between arrows) in *V. formosa.* Note chromosome figures (CH) in cell at left.

Figure 5. Cluster of spermatocytes with densely staining nuclei (N), small cell extensions (arrows), and mitochondria (M) in *V. formosa.*

Figure 6. High magnification of intercellular bridge between adjacent *V. formosa* spermatocytes. Note numerous microtubules (MT) and electron dense thickening of bridge plasmalemma (arrows).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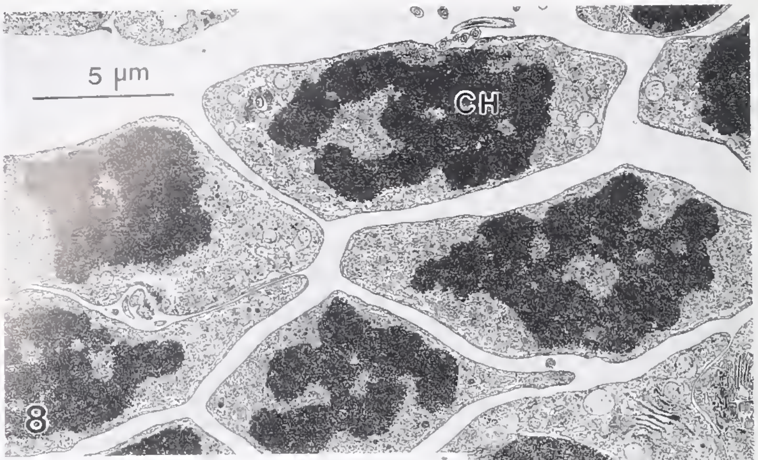
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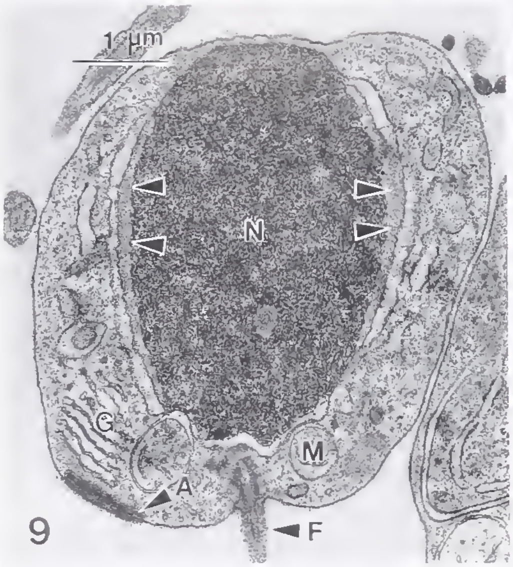
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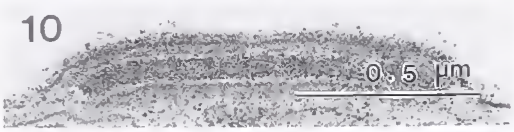
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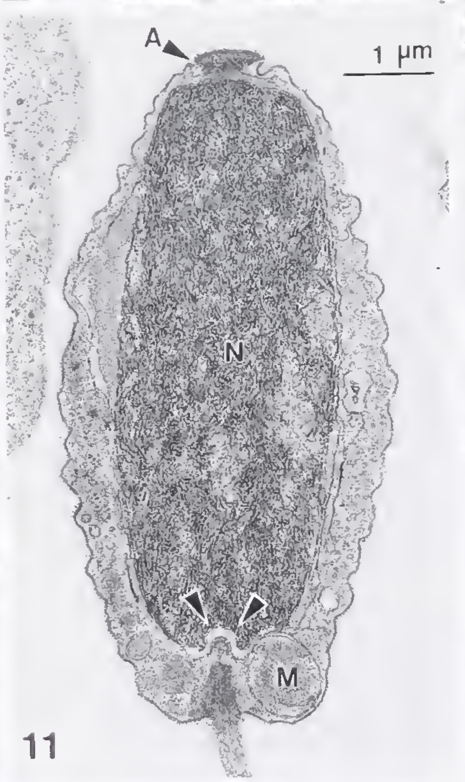
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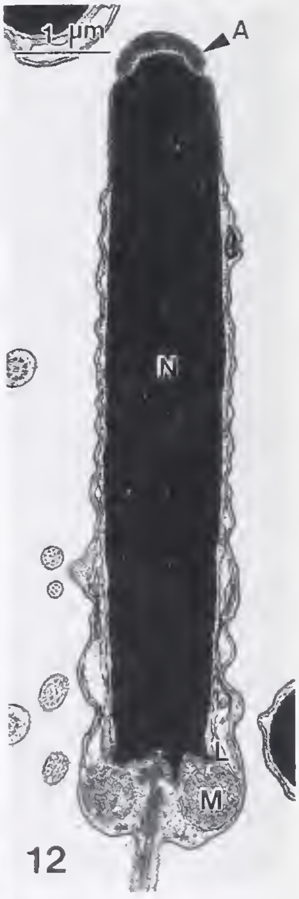
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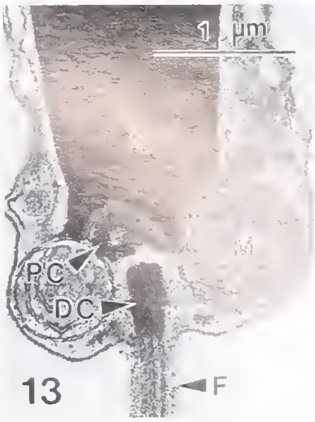
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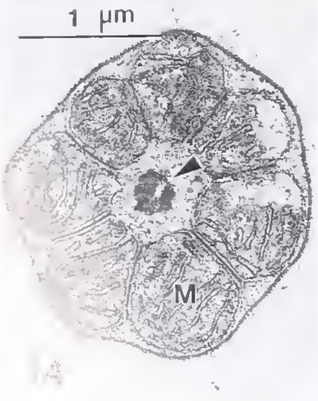
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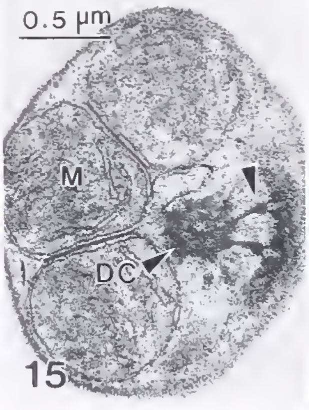
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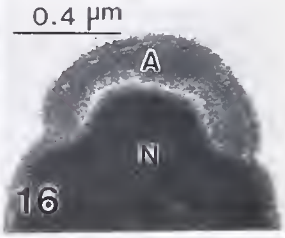
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epithelium and undergo numerous mitotic divisions in the coelom followed by meiotic divisions and terminal differentiation of peripheral cells in the larger cell clusters. Spermatids detach from these cell clusters and mature singly in the coelom in *V. formosa* or in groups in *K. lepidota*. The designation of spermatogenic stages in the following account is based upon the size of cell clusters, size and morphology of nuclei, and the appearance of characteristic structures such as centrioles and flagellum.

The following account of spermatogenesis is based primarily upon specimens of *V. formosa* for which the most material was available. The sequence of events and the ultrastructure of sperm development are very similar in *V. formosa* and *K. lepidota*. It may be assumed that the following descriptions apply to both species unless otherwise specified. Cellular measurements refer to *V. formosa* unless specifically attributed to *K. lepidota*.

Proliferating spermatogonia

The earliest stages encountered in the coelom are small spermatogonial clusters of 8–10 cells that are surrounded by sheath cells presumably derived from the coelomic peritoneum (Fig. 3). In some instances, several small clusters of proliferating cells are surrounded by a common cellular envelope of sheath cells. The nuclei of these spermatogonial cells are irregular in shape with an average diameter of 7.2 μm (n = 37). The chromatin is granular and heterogeneous in non-dividing nuclei (Fig. 4). Chromosome figures are present within the nuclei of some cells (Fig. 4). In later proliferating stages, the nuclei become more electron-dense and more spherical in shape (Fig. 5). Cytoplasmic volume is about equal to, or less than, nuclear volume during interphase with prominent intercellular bridges joining up to four adjacent cells. Intercellular bridges are characterized by a collar of electron-dense material and by the presence of numerous microtubules crossing the bridges along the axial plane (Fig. 6). Two centrioles, oriented perpendicular to each other are present near intercellular bridges (Fig. 7). Numerous mitochondria are present throughout the cytoplasm along with well-developed Golgi cisternae (Figs. 5, 7).

Repeated mitotic divisions produce coelomic cell clusters up to 0.8 by 0.4 mm that contain approximately 800 cells. Nuclear diameter averages 5.9 μm (n = 30) in these cells. The sheath cells are absent after the early stages of proliferation although cytoplasmic extensions of peripheral cells in larger clusters gives the appearance of a sheath cell covering (Fig. 5).

Spermatocytes and early spermatids

Chromosome figures are commonly observed in cells at the periphery of larger spermatogonial cell clusters (Fig. 8). These peripheral cells become detached from the cluster and lose cytoplasmic continuity with adjacent cells. It is likely that these dividing cells represent primary and secondary spermatocytes undergoing meiosis.

In *Vanadis formosa*, cytoplasmic bridges are infrequently encountered between cells that have detached from the large cell clusters. However, in *Krohnia lepidota*, groups of four spermatids are connected by cytoplasmic bridges. These groups of four cells are likely the result of the two meiotic divisions of spermatogonial cells.

Following the presumptive meiotic divisions, the nuclei become spherical again with an average diameter of 3.9 μm (n = 3). The chromatin of these early spermatids

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Figure 7. Intercellular bridge (between arrows) in *Vanadis formosa* with adjacent centrioles (C) and Golgi complex (G) in developing spermatocytes.

Figure 8. Chromosome figures in dividing *V. formosa* spermatocytes.

Figure 9. Early *V. formosa* spermatid with oval nucleus (N) containing granular chromatin and lateral regions of uncondensed nucleoplasm (arrows). Note Golgi complex (G) and adjacent proacrosome (A), mitochondrion (M), and flagellum (F).

Figure 10. Higher magnification of proacrosome from Figure 9.

Figure 11. Elongating spermatid of *V. formosa* showing filamentous chromatin in nucleus (N), developing nuclear fossa at base of nucleus (arrows), and terminal acrosome (A).

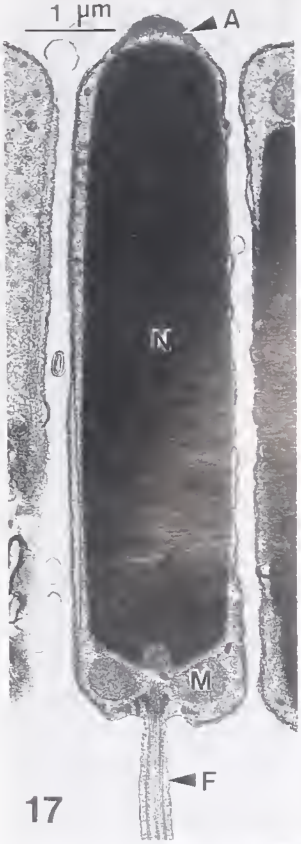
Figure 12. Mature spermatozoan of *V. formosa*. A, acrosome; N, nucleus; M, mitochondrion.

Figure 13. Higher magnification of middlepiece region of mature spermatozoan. Note the asymmetry of the posterior region of the nucleus (N). DC, distal centriole; PC, proximal centriole; F, flagellum; M, mitochondrion.

Figure 14. Cross section through middlepiece of mature *V. formosa* spermatozoan showing ring of mitochondria (M) and centrally positioned distal centriole (arrow).

Figure 15. Tangential section through middlepiece of mature *V. formosa* spermatozoan showing mitochondria (M) and distal centriole with a portion of the centriolar anchoring devise (arrows).

Figure 16. High magnification of acrosome (A) and anterior portion of the nucleus (N) in *V. formosa* mature spermatozoan.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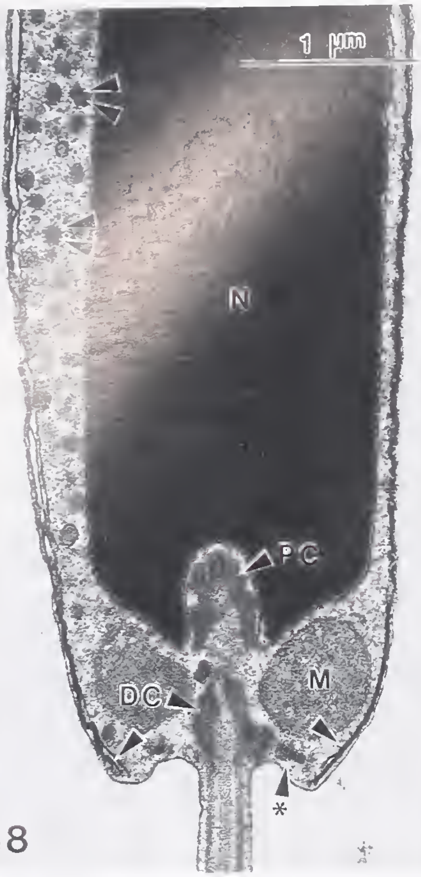
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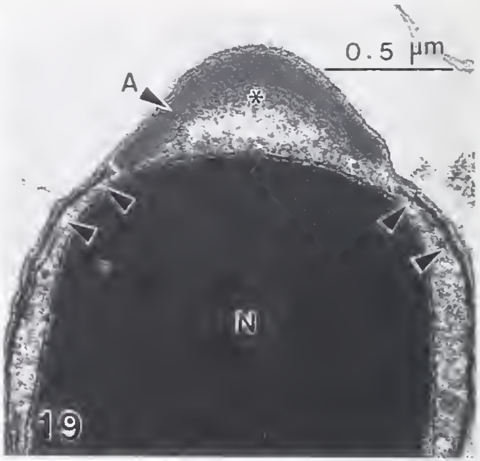
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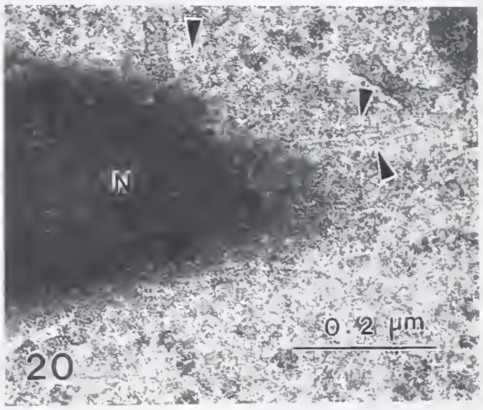
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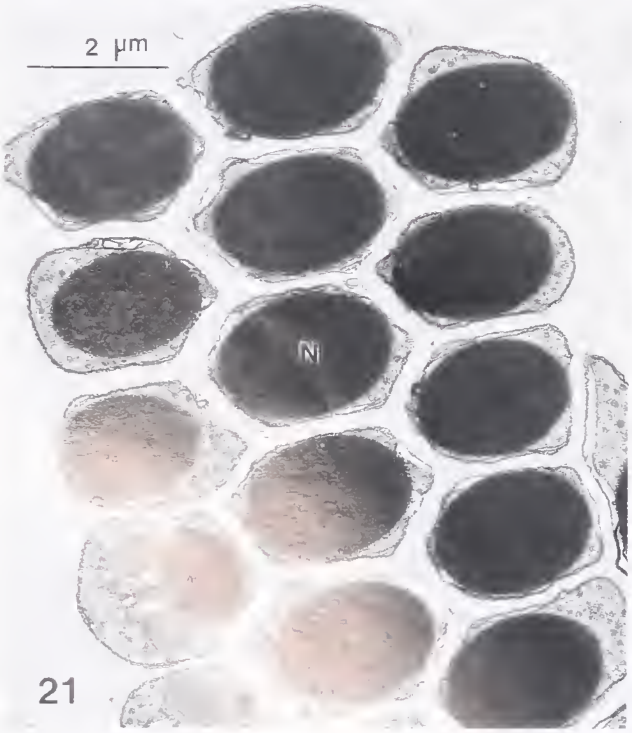
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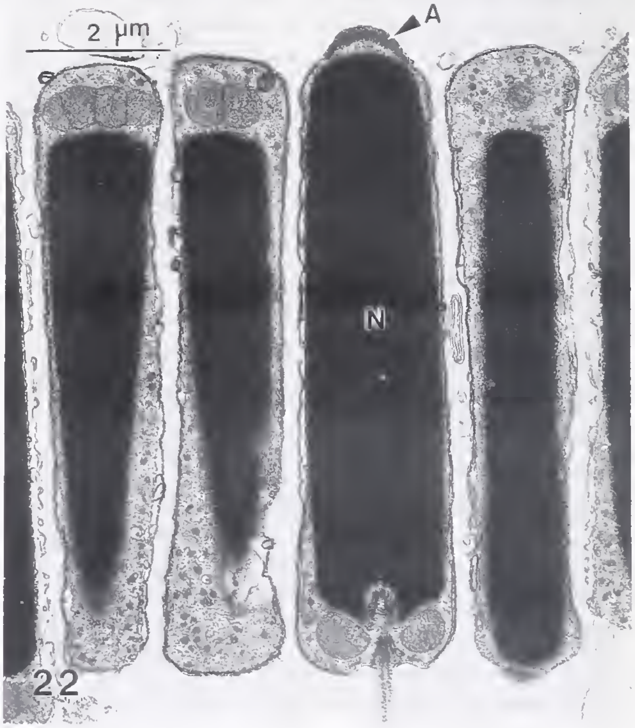
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appears coarsely granular with mottled patches of electron-transluscent material especially along the peripheral region of the nucleus (Fig. 9). Two centrioles are present and the sperm flagellum begins to form signaling the beginning of terminal differentiation of the cell. Four to seven large mitochondria accumulate at the posterior end of the spermatids prior to nuclear elongation and surround the developing centriolar complex of the flagellum (Fig. 9). The proacrosome and a single Golgi complex are located in the posteriolateral portion of the cell near the flagellum (Fig. 9). The proacrosome is membrane-bounded and closely applied to the plasma membrane of the cell. Its contents are homogeneous and electron-dense (Fig. 10).

Late spermatids

The most conspicuous events that distinguish early from late spermatids are the location of the proacrosomal vesicle, elongation of the nucleus, formation of the centriolar fossa at the base of the nucleus, and a textural change in the chromatin from granular to fibrillar in late spermatids.

The proacrosomal vesicle is positioned at the anterior end of the sperm while maintaining close contact with the plasmalemma. The vesicle is plate-like in shape with a flattened appearance in cross section (Figs. 10, 11). The Golgi complex that was initially associated with the acrosomal vesicle is no longer visible.

The nucleus elongates progressively reaching a length of 5.6 μm when the proacrosome reaches the apex of the spermatid. In favorable sections, a manchette of microtubules surrounds the spermatid nucleus. At the posterior end of the nucleus, a symmetrical fossa containing the proximal centriole forms (Fig. 11).

Chromatin condensation is characterized by the appearance of electron-dense fibers interspersed with patches of less-dense nucleoplasm. In cross section, the chromatin fibers measure 30 nm and are generally oriented parallel to the long axis of the nucleus (Fig. 11). An inflated nuclear envelope (or perinuclear cisternae, from the older literature) is characteristic of this stage. The chromatin fibers become progressively more densely packed and thus electron-opaque as the nucleus completes elongation.

Mature coelomic sperm of Vanadis formosa

The mature coelomic sperm (Fig. 12) has a homogeneously electron-dense nucleus with an average length of 7.7 μm (n = 6). The nucleus is slightly fusiform in shape with a diameter of 1.4 μm (n = 6) near the midpoint. The acrosome forms a cap, about 1 μm in diameter, over the tip of the nucleus (Fig. 16). A constriction at the anterior tip of the nucleus forms a ledge upon which the acrosome rests giving the anterior end of the sperm a smooth to slightly indented outline (Fig. 16). Nuclear material extends into the convex space below the acrosome. The acrosomal contents are homogeneous and less electron-dense than the nucleus. The acrosome is bounded by an acrosomal membrane and overlain by the closely apposed sperm plasma membrane. The plasmalemma has an irregular outline over all but the most anterior region of the cell, which may represent a fixation artifact (Fig. 12). The posterior portion of the nucleus is irregularly shaped with a displaced centriolar fossa measuring 1.2 by 1.0 μm and containing the proximal centriole (Fig. 13).

The middlepiece is short compared to the nucleus with an average length of 1.0 μm (n = 5) and contains 5–7 spherical mitochondria surrounding the centriolar complex at the base of the nucleus (Fig. 14). Mitochondrial diameter averages 0.7 μm (n = 2) with a maximum diameter of 0.9 μm. The centriolar complex is composed of a proximal centriole located at the base of the nucleus and a distal centriole located between the mitochondria and continuous with the sperm flagellum (Fig. 13). The proximal centriole contains nine triplets of microtubules and

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Figure 17. Mature spermatozoan of *Krohnia lepidota*. A, acrosome; N, nucleus; M, mitochondrion; F, flagellum.

Figure 18. Posterior half of mature *K. lepidota* spermatozoan showing proximal centriole inserted into symmetrical fossa at base of nucleus (N). Note accessory membranes beneath lateral plasmalemma (single arrows) and electron dense material (*) associated with the distal centriole (DC) posterior to the mitochondria (M). The sperm cytoplasm contains numerous membrane-bounded inclusions (double arrows).

Figure 19. Anterior region of mature *K. lepidota* spermatozoan showing acrosome (A), subacrosomal material (*), and accessory membranes (double arrows) beneath the plasmalemma. N, nucleus.

Figure 20. Tangential section through nuclear region of *K. lepidota* spermatozoan showing microtubular manchette (arrows) running parallel to the long axis of the nucleus (N).

Figure 21. Cross section through coelomic sperm packet in *K. lepidota*. Note regular spacing between adjacent sperm.

Figure 22. Longitudinal section through coelomic sperm packet in *K. lepidota* Note the alternating orientation of sperm in the packet.

is positioned perpendicular to the long axis of the sperm in a shallow centriolar fossa. The distal centriole also contains nine triplets of microtubules as well as nine lateral, branching extensions forming an anchoring structure at the base of the flagellum (Fig. 15).

Mature coelomic sperm of Krohnia lepidota

Although the general shape of the coelomic sperm is similar between the two species, the mature coelomic sperm of *Krohnia lepidota* differ from those of *Vanadis formosa* in size, organelle shape, cytoplasmic inclusions, and the presence of accessory membranes. The head of the mature coelomic sperm of *K. lepidota* (Fig. 17) measure 11.0 μm (n = 5) in length (acrosome + nucleus + middlepiece). The acrosome is cap-like with a diameter of 1.2 μm (n = 10) and a subacrosomal space filled with a flocculent material (Fig. 19). In favorable sections, a manchette of microtubules is observed parallel to the nucleus (Fig. 20). The nucleus measures 7.7 μm in length (n = 7) with a maximum diameter of 1.7 μm (n = 22). A thin layer of cytoplasm surrounds the nucleus and middlepiece and contains numerous spherical, membrane-bounded inclusions of variable electron density and unknown composition (Fig. 18). These inclusions range in size from 0.08–0.14 μm (mean = 0.11, n = 14).

The middlepiece is short (ca. 2 μm) as in *Vanadis formosa*, and contains 8–10 spherical mitochondria that average 0.7 μm in diameter (n = 10). A centriolar fossa extends about 0.5 μm into the posterior portion of the nucleus and contains the proximal centriole (Fig. 18). The distal centriole is located between the mitochondria and is attached to a nine-spoke flagellar anchoring structure similar to that of *Vanadis formosa* described above (Fig. 18).

The nucleus and middlepiece are partially surrounded by a series of electron-dense accessory "membranes" lying just beneath the sperm plasmalemma (Figs. 18, 19). The "membranes" appear to be composed of a layer of electron-dense material and are absent from the extreme anterior and posterior regions of the sperm head (Figs. 18, 19). They are thicker and stain more densely than the nuclear envelope or the plasmalemma. As many as three "membranes" are present in some sperm between the plasmalemma and the nuclear envelope.

The coelomic sperm of *K. lepidota* often occur in packets of 18 or more cells oriented parallel to each other (Fig. 21, 22). No cytoplasmic connections are visible between cells to account for the apparent cohesiveness of these packets. In longitudinal sections, the sperm alternate with regard to anterior-posterior orientation (Fig. 22).

Hermaphroditic Vanadis formosa

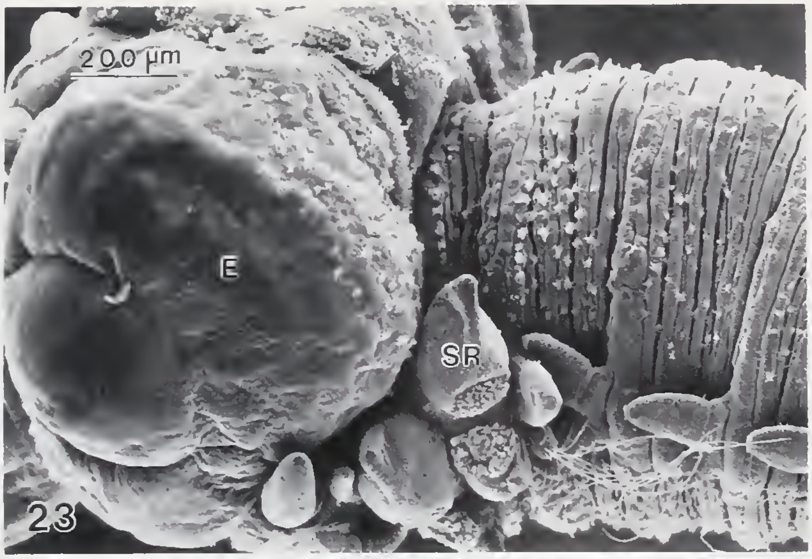
Two out of 59 specimens of *Vanadis formosa* examined contained both sperm and eggs within the coelomic compartment. Numerous stages of sperm development from early spermatogonia to late spermatids were present while egg development appeared to be in early stages only. Egg diameters ranged from 31 to 78 μm, corresponding to the previtellogenic and early vitellogenic stages of other alciopid polychaetes. Both sperm and eggs appear to be normal with no signs of gamete resorption that might indicate a progressive sex reversal.

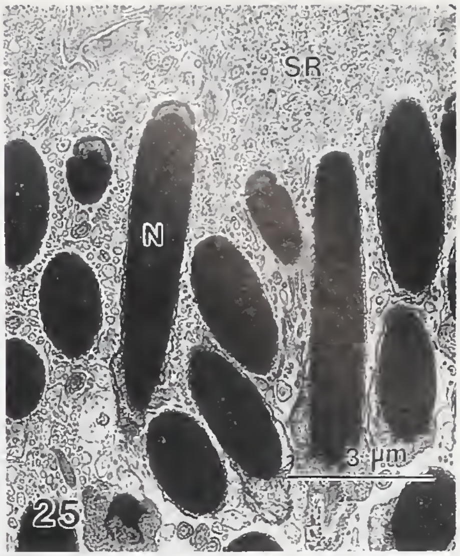
Sperm storage by females

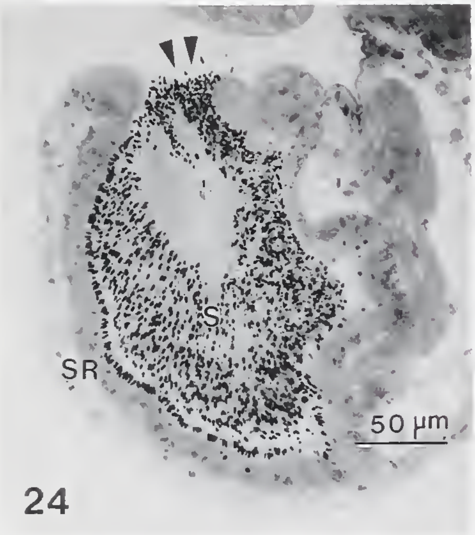
Vanadis formosa females store sperm within modified dorsal cirri of the first and second post-head segments (Figs. 2, 23, 24). A small pore on the ventral surface of the receptacle serves as the entry and exit site for the sperm (Fig. 24). The wall of the receptacle appears to be composed of two cell layers (Fig. 24) with the sperm heads embedded in the inner cell layer (Fig. 25). The sperm within these seminal receptacles are morphologically similar to what we consider to be mature sperm within the coelom of the males. The acrosomes of stored sperm are unreacted while the nuclear region is surrounded by numerous microvilli extending from maternal cells (Fig. 26). No intercellular junctions are visible between maternal tissues and sperm. The number of sperm contained within seminal receptacles varies between individual females and with the size of the storage organ. The ultrastructural details of sperm storage in alciopid polychaetes will be reported elsewhere.

Discussion

Vanadis formosa and *Krohnia lepidota* are among the most commonly encountered cosmopolitan alciopids in tropical and temperate seas (Tebble, 1960, 1962; Day, 1967; Dales and Peter, 1971). However, intact specimens suitable for TEM studies are difficult to obtain due to the fragile structure of most alciopids. These animals have not been successfully maintained in the laboratory and thus unanswered questions remain concerning their biology. For example, many alciopids, including *V. formosa* and *K. lepidota*, are transparent when alive but have never been observed to be full of gametes. The coelomic cavity is spacious with relatively few gametes present, relative to available space, as compared to other polychaetes of comparable size. This may be due to a sampling artifact in the number of individuals collected or may represent an adaptive strategy for increased transparence (see below). The length of the gametogenic cycle and the possibility of consecutive hermaphroditism are







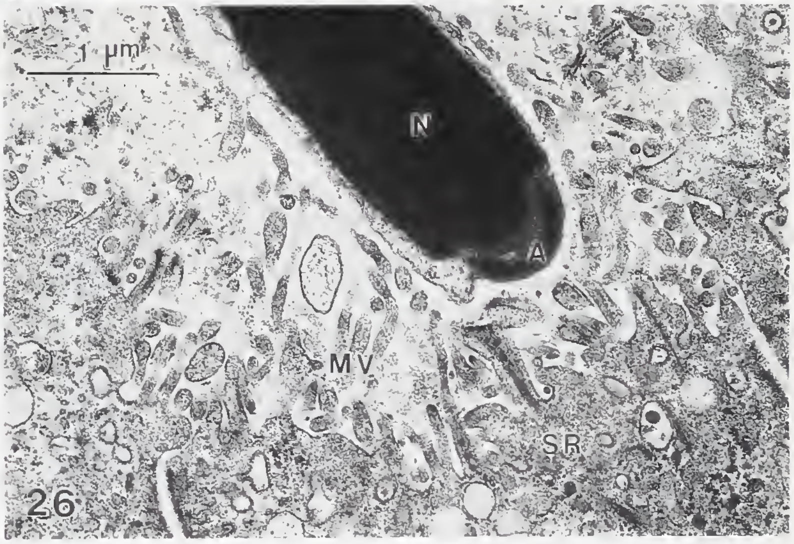


Figure 23. Scanning electron micrograph showing lateral view of head in female *Vanadis formosa.* Note the enlarged dorsal cirri of the first two podia, just behind the eye (E), that serve as seminal receptacles (SR).

Figure 24. Light micrograph of a one micron thick section through one of the seminal receptacles (SR) showing enclosed sperm (S) and pore leading to the exterior (double arrows).

Figure 25. Longitudinal section of mature *V. formosa* sperm embedded in the wall of the seminal receptacle. Note that the sperm nuclei (N) are oriented at various angles within the cells of the receptacle wall (SR).

Figure 26. Higher magnification of sperm nucleus (N) and acrosome (A) surrounded by microvilli (MV) of the seminal receptacle cells (SR). Note that the sperm acrosome is unreacted and that there appear to be no cell junctions between sperm and maternal cells.

also difficult to analyze without laboratory data on living specimens.

The sperm of alciopids vary in general morphology from short, fusiform "primitive" ones (Franzén, 1956) to elongated types more characteristic of "modified" sperm (Rice, 1987). Both *V. formosa* and *K. lepidota* have sperm with elongated nuclei and short middlepieces. In size and general morphology, the sperm of these two species appear very similar suggesting that (1) *Vanadis* and *Krohnia* are closely related genera or (2) convergent evolution of sperm structure has occurred. Rice (1987) applied a Pearson product-moment correlation coefficient analysis to the nine alciopid genera and found that *Vanadis* and *Krohnia* were not closely related (similarity less than 25%) based upon ten morphological and reproductive characteristics. It is likely that the similarity of the sperm in these two species is more closely related to reproductive biology than to recent common ancestry.

The primordial germ cells and gonads of most polychaetes are associated with the coelomic epithelium or the intestinal/nephridial blood vessel epithelium (Olive, 1983; Eckelbarger, 1984; Sawada, 1984). In *Vanadis formosa* and *Krohnia lepidota*, no gonads or primordial germ cells were observed in these traditional locations. In the alciopids *Rhynchonerella angelini* and *Alciopa reynaudii*, Eckelbarger and Rice (1988) found no definitive ovary and reported that the earliest stages observed were coelomic oogonia.

There is a high degree of similarity in sperm development between the families Nereidae, Phyllodocidae, and Alciopidae, which is consistent with their traditional systematic grouping (Fauchald, 1977). In the phyllodocid *Eulalia viridis*, Olive (1975) reported that a "dispersed gonad" exists in which free-floating coelomic cells of uncertain origin give rise to the gametes. These coelomic germ cells then produce large packets of spermatogonia, containing thousands of cells, and eventually disperse as rosettes of primary and secondary spermatocytes. In the Nereidae, the location of definitive gonads has also proven illusive (Eckelbarger, 1984; Sawada, 1984). The earliest spermatogenic stage reported for *Perinereis brevicirris* was coelomic cell clusters of several hundred spermatogonia that appeared to be subdivided by cytoplasmic processes into blocks of several dozen cells each (Kubo and Sawada, 1977). In *Platynereis dumerilii* and *P. massiliensis*, proliferating spermatogonia appear from an unidentified germinal epithelium as free-floating clusters of 60 or more cells that are enveloped by sheath cells (Pfannenstiel *et al.*, 1987). The development of spermatogonial clusters within the coelomic cavity is not unique to these three families however, with similar processes reported for *Pomatodrilus fluviatilis* (Bunke, 1985) and for *Nicolea zostericola* (Eckelbarger, 1975) although in both of these species, the germ cells could be traced back to a distinct gonad.

The sequence of the meiotic and mitotic divisions during sperm development could not be established with certainty in the present study. However, in other polychaetes the presence of synaptonemal complexes or other indicators of meiosis have been reported to occur as peripheral cells detach from coelomic spermatogonial clusters (Olive, 1975; Kubo and Sawada, 1977; Sawada, 1984). It is likely that this occurs in *V. formosa* and *K. lepidota*.

Spermiogenesis in *V. formosa* and *K. lepidota* is similar to that described for other polychaetes. The condensation of chromatin follows the fibrillar model and the nucleus is surrounded by a manchette of microtubules (see Sawada, 1984, and Franzén and Rice, 1988, for reviews). It has been suggested that the microtubules associated with the nucleus during condensation and elongation in *Spirorbis morchi* are directly involved in shaping the nucleus (Potswald, 1967). In other polychaete species however, nuclear condensation and elongation proceed without the assistance of microtubules (Rice, 1981).

The acrosomes of *V. formosa* and *K. lepidota* are typical of the Alciopidae and relatively simple in structure. With the exception of *Torrea candida*, which has a pointed conical acrosome (Rice, 1987), and *Alciopina parasitica*, which has a complex elongated acrosome (Rice, unpub. obs.), all alciopid species examined have a simple cap-like acrosome similar to *V. formosa* and *K. lepidota* (Rice, 1984, 1987). In *Eulalia* sp., the only phyllodocid for which ultrastructural information is available, the acrosome is simple and cap-like (Rouse, 1986, 1988).

The irregular shape of the posterior nucleus in mature *Vanadis formosa* sperm is relatively unusual in polychaetes. Typically, the posterior portion of the nucleus is rounded with an implantation fossa containing the centriolar complex (Sawada, 1984; Franzén and Rice, 1988). It is not uncommon for the posterior nucleus of polychaete sperm to have symmetrical indentations where it rests against the mitochondria as in *Naiades cantrainii* and *Branchiomma bombyx* (Franzén and Rice, 1988). The irregular shape of the posterior nucleus in *V. formosa* imparts an asymmetry to the sperm with respect to the centriolar complex and flagellum. This asymmetry would likely cause the sperm to rotate while swimming. A similar asymmetry has been reported in the sperm of *Exogone gemmifera* (Franzén, 1956), *Chitinopoma serrula* and *Capitella capitata* (Franzén, 1982; Eckelbarger and Grassle, 1987), and *Phragmatopoma lapidosa* (Eckelbarger, 1984). The offset flagellum in *P. lapidosa* may counter the effects of the elongated, curved acrosome to stabilize the sperm during swimming (Eckelbarger, 1984).

The accessory "membranes" observed in the sperm of *Krohnia lepidota* are unique within the Polychaeta. Franzén (1982) observed electron-dense layers between the nucleus and plasmalemma of *Autolytus* sp. sperm but determined these to be flagellar rootlets eminating from the centriolar complex. He suggested that these rootlets functioned in anchoring the flagellum to the sperm cell. The accessory membranes in *K. lepidota* sperm may represent an artifact of fixation, although we have seen no comparable structures in dozens of other polychaete species fixed under similar conditions.

Olive (1983) and Franzén and Rice (1988) have reviewed the hypothesis that elongated or modified sperm in the Polychaeta are associated with specialized mechanisms of sperm transfer, sperm storage, or fertilization biology. In the Alciopidae, modified as well as primitive sperm types are found (Rice, 1987). Rice (1984) classified the sperm of both *V. formosa* and *K. lepidota* as modified based upon their elongated nuclei. Sperm storage in specialized seminal receptacles is known for *V. formosa* and the elongation of the sperm may represent an adaptation for sperm storage as in spionid polychaetes (Rice, 1981). However, in *K. lepidota*, Stöp-Bowitz (1948) reported that sperm may be carried between pedal lobe and ventral cirrus on the exterior of the female. No mature females were collected in the present study and thus sperm storage in *K. lepidota* could not be confirmed. Eckelbarger and Rice (1988) and Rice (1987) have confirmed external storage of sperm in the alciopids *Rhynchonerella angelini* and *R. moebii*, although both species have short, primitive sperm. Two other genera of alciopids, *Torrea* and *Naiades*, have specialized internal sperm storage organs in the females while sperm morphology is basically of the primitive type.

The ability of female worms to store sperm has been viewed as a possible adaptation to pelagic life in the Alciopidae (Rice, 1987). Six of the nine known genera in this family display some form of sperm storage in the female. With relatively low population densities (Rice, pers. obs.) and vast amounts of available habitat, it would seem advantageous for these worms to be able to take advantage of infrequent sexual encounters through transfer and storage of sperm. An evolutionary sequence of sperm storage can be envisioned leading from broadcast spawning without sperm storage, (phyllodocids), to external sperm storage of "primitive type" sperm on females, (*Rhynchonerella*), to internal storage of "primitive type" sperm, (*Torrea* and *Naiades*), and finally to internal storage by females of modified sperm, (*Vanadis*). This sequence suggests that other pelagic animals that arose from a benthic, broadcast-spawning stock might tend toward modified sperm structure and sperm storage as increasing adaptations to pelagic life.

An alternative method of minimizing the problem of infrequent sexual encounters in the open ocean would be to develop hermaphroditism (Harbison and Miller, 1986). Out of 110 alciopids representing 13 species, we have encountered only two specimens, both *V. formosa*, that were simultaneous hermaphrodites. The possibility of consecutive hermaphroditism in the Alciopidae exists, but perhaps the development of sperm storage mechanisms has been the major evolutionary thrust within the family to overcome their suspected infrequent encounters with potential reproductive partners.

Acknowledgments

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Rapid Evolution of Metal Resistance in a Benthic Oligochaete Inhabiting a Metal-polluted Site

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Abstract. We identified a case of very rapid evolution of resistance in a common freshwater benthic invertebrate, to sediment with extremely high levels of cadmium and nickel. *Limnodrilus hoffmeisteri* from metal-polluted sites in Foundry Cove (New York) was significantly more resistant than conspecifics from a nearby control site, to both metal-rich natural sediment and metal-spiked water. Resistance differences were also found among sites within Foundry Cove. The elevated resistance in Foundry Cove worms was genetically determined, as it was still present after two generations in clean sediment. Resistance had evolved rapidly (within 30 years). A laboratory selection experiment and estimates of the heritability of this resistance in *L. hoffmeisteri* from the control site, indicated that the resistance could have evolved in 1 to 4 generations. The laboratory selection resulted in a large increase in resistance after two generations of selection, while we demonstrated that most of the phenotypic variation was additive genetic; heritability estimates ranged from 0.59 to 1.08.

Introduction

Environmental pollutants may exert strong selective pressures on natural populations, offering unique opportunities for studying natural selection. The selective agent, selection strength, and the timing of the selection process are often known and quantifiable, while the selective pressures may be so strong that effects become evident rapidly. In addition, the evolution of resistance to pollutants can illustrate the processes involved in early stages of speciation (Macnair, 1987). But considerations of a less theoretical nature also underscore the importance of investigating the evolution of resistance. If populations inhabiting polluted sites commonly evolve a resistance to the toxicants present, long-term effects of pollutants on natural populations will be very different from those predicted from bioassays (Levinton, 1980).

The evolution of resistance to metals is well documented for plants (Antonovics *et al.*, 1971; Klerks and Weis, 1987; and Macnair, 1987), but very little evidence is available demonstrating metal adaptation in natural populations of animals (Klerks and Weis, 1987). However, it is known that genetic variation for metal resistance is present in several populations of aquatic animals (*e.g.*, Lavie and Nevo, 1982; Nevo *et al.*, 1984; and Lavie and Nevo, 1986). And an elevated metal resistance is reported for many populations of aquatic animals (Klerks and Weis, 1987). But the presence of a genetic basis for these resistance differences among populations from differently polluted environments is confirmed only by Brown (1976) for a population of isopods inhabiting a metal-polluted river in S.W. England. In addition, there is some evidence that the increased resistance in the polychaete *Nereis diversicolor* from several metal-polluted rivers in S.W. England (Bryan and Hummerstone, 1971, 1973; Bryan, 1976) might be genetically determined, as de-acclimation of copper-resistant worms did not result in a reduced resistance (Bryan and Hummerstone, 1971; Bryan and Gibbs, 1983).

Most polluted areas contain many different toxicants. This complexity hinders an investigation into the evolution of resistance, as this makes it difficult to pinpoint the selective force(s). An unusual situation exists in Foundry Cove, New York (a freshwater bay on the Hudson River). This tidal cove and marsh has elevated levels of

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a very limited set of pollutants. Foundry Cove received wastewater rich in Cd, Ni, and (at times) Co from a battery factory, during the period 1953–1971 (Resource Engineering, 1983). Sediment Cd levels as high as 50,000 μg Cd per g dry sediment were reported for 1975 (Kneip and Hazen, 1979). More recent findings showed decreased Cd concentrations in the surface layer of the sediment, though much sediment still contains over 500 μg/g Cd. An extremely high Cd level (225,000 μg/g) was encountered in 1983 close to the original outfall site at a depth of 10–20 cm (Knutson *et al.*, 1987). Concentrations of Cd, Ni, and Co are highly correlated, and the three metals occur in molar ratios of 18:20:1, respectively (Knutson *et al.*, 1987). Benthic macrofauna taken from this sediment and allowed to exclude their gut contents, have elevated body burdens of both Cd and Ni (Klerks, 1987). This demonstrates that the Cd and Ni in Foundry Cove sediment are bioavailable. Nevertheless, organisms are present in this sediment. Overall densities of macrobenthos in Foundry sediment are similar to those of the control area, though there is evidence for a reduction of the taxonomic diversity at the most polluted site (Klerks and Levinton, 1989).

We investigated the evolution of resistance to a combination of Cd, Ni, and Co in the deposit feeding annelid *L. hoffmeisteri.* This tubificid oligochaete is the most common macrobenthic species at both Foundry Cove and a nearby control site (Klerks, 1987). The evolution of metal resistance was approached in two ways. To determine if *L. hoffmeisteri* from Foundry Cove was genetically adapted to the metals, we compared the resistance of these worms and their offspring to the resistance in conspecifics from the control site. Our results showed that *L. hoffmeisteri* from Foundry Cove was genetically adapted to the pollutants. To find out how likely it was that such an adaptation would occur again and to confirm our evidence for a very rapid evolution of resistance, we determined the heritability of the metal resistance in the control population and performed an experiment in which control worms were selected for an increased resistance. These experiments showed that the control population had the potential for a rapid evolution of resistance to a combination of Cd, Ni, and Co.

Materials and Methods

Animal collections and laboratory cultures

Both *L. hoffmeisteri* and sediment were collected by Ekman grab in either Foundry Cove (near Cold Spring, New York, and 87 km upriver from the southern tip of Manhattan) or the control area (South Cove, about 2 km south of Foundry Cove). Worms were sorted from the >500 μm fraction of sediment. Laboratory cultures were set up in polystyrene dishes, with a 1-cm layer of sediment and 9 cm of continuously aerated Hudson River water. The sediments for the cultures were collected from the same sites as the animals, sieved (to <500 μm), boiled, washed with GF-C filtered Hudson River water, frozen, and thawed shortly before use. Hudson River water was collected near the control area and filtered with a GF-C glassfiber filter prior to use. Ground fishfood flakes were added to the cultures once per week unless previously added food was still present. Cultures were kept at 24°C under a 13 h light/11 h dark cycle.

To obtain the second generation of unexposed offspring, Foundry Cove worms were cultured in sediment from the control area (containing 19 μg/g Cd), for two generations. Newborn juveniles were separated from the adults approximately monthly, to keep the different generations apart.

L. hoffmeisteri is a simultaneous hermaphrodite, reproducing sexually by crossfertilization, though uniparental reproduction has been reported (Gavrilov, 1931). Autotomy and fragmentation followed by regeneration are common, but do not result in additional individuals (Kennedy, 1966).

Resistance comparisons

Experiments comparing the toxicity of metal-rich sediments to *L. hoffmeisteri* from Foundry Cove and the control site were done in glass beakers (10 cm diameter), containing a 1-cm layer of sediment and 9 cm of aerated overlying water. Water and sediment were treated similarly as for the cultures, except that these sediments had been sieved to a <250 μm size to facilitate detection of newborn juveniles. These bioassays were performed with natural sediments from Foundry Cove and the control area. The experiments were started with ten adult worms per replicate and three replicates per group. Ground fishfood flakes (0.05 g) were added at the beginning of the experiment and after 14 days. The number of survivors (and newborn juveniles) were determined after 28 days. The first experiment using this experimental procedure was performed with worms collected from Foundry Cove (from a site with approximately 7000 μg Cd per g dry sediment) and from the control area (19 μg/g Cd). The same procedure was used in a second experiment to investigate the presence of resistance differences between worms collected from the control area, worms from Foundry Cove, and the second generation offspring of Foundry Cove worms.

Metal concentrations of sediments were determined by flame atomic absorption spectrophotometry. Samples were homogenized, dried at 70°C, and ground to a powder. Small aliquots (<1 g) were filtered after refluxing with 7.5 ml each of nitric and hydrochloric acids (reagent grade) for 5 h at 120°C. The filtrate was brought up to 50 ml with distilled water and analyzed with an Instrument Laboratory Model 157 spectrophotometer.

A second type of bioassay involved the determination of survival times in metal-spiked water. Mature *L. hoffmeisteri* were exposed to a solution containing 8.9 μM Cd, 10.2 μM Ni, and 0.5 μM Co (nominal concentrations using chloride salts), in soft reconstituted fresh water (ASTM, 1980). (The specific metal concentrations were chosen arbitrarily, though the ratios for the three metals were the same as in Foundry Cove sediments). The water was aerated prior to use and animals were acclimated overnight to this water. Both acclimation and exposure took place in plastic Petri dishes (100 × 15 mm) with 40 ml reconstituted water or metal solution at an initial density of 6 worms per dish. Animals were transferred to new dishes (the 6 worms from an acclimation dish were transferred together) after the acclimation period. At time zero, 0.4 ml of a solution containing 100 times the experimental metal concentrations was added to 39.6 ml water. Experimental control animals were treated similarly as the exposed animals except that no metal stock solution was added. The animals were not fed during either acclimation or exposure. Survival was determined every hour. The experiment was terminated when all the exposed animals had died. Actual metal concentrations in solution were not determined, since the purpose of these experiments was entirely comparative. The first experiment using this type of bioassay was performed with worms from the control area and from three differently polluted sites in Foundry Cove (7 replicate dishes with 6 worms each per group). Subsequent experiments using this procedure were performed on groups of worms from selection lines (usually 10 replicate dishes with 6 worms each per group, see below) and on control area worms for the heritability determinations (with 1 to 6 worms per dish, see below).

Selection experiments

The starting population was initiated by combining control area *L. hoffmeisteri* from several laboratory cultures. Worms from the different cultures were divided equally over four lines (three selection lines and a control line), resulting in 441 juveniles for each of the lines. In selecting the first generation, 200 adults from each of the three selection lines were exposed to Cd-rich sediment (approximately 50,000 $\mu g/g$ Cd) until 80 to 90% of the worms had died. The surviving adults were then transferred to reproduce in dishes with control area sediment. The ultimate number of survivors was determined after three months and these were transferred again to clean sediment. The latter was done to exclude juveniles born from mating occurring prior to the final selection of the parent generation, since spermatophores may remain in spermatheca for up to six weeks (Kennedy, 1966). The next generation was later started with 500 of their offspring per line, in clean sediment.

The selection procedure for subsequent generations was modified to achieve a shorter time between generations. Worms with not fully developed gonads were exposed to a combination of 8.9 μM Cd, 10.2 μM Ni, and 0.5 μM Co in soft reconstituted fresh water for a duration aimed at an ultimate survival of 25%. Initial survivors were transferred to culture dishes with clean sediment. These worms were checked monthly to determine ultimate survival and to remove 500 juveniles (per line) for the next generation.

For the control line, the number of adults was reduced to achieve a similar inbreeding effect as in the selected lines, using the harmonic mean of the population sizes in successive generations to determine the mean effective population size and the mean rate of inbreeding (Falconer, 1981).

The selection process was continued for three generations. The proportion of the population selected ranged from 7.5 to 29% (mean: 14%).

The resistance of each line in each generation was quantified by determining survival times in the above-described bioassay with Cd, Ni, and Co in reconstituted water. Survival times were generally determined on 60 mature worms per line. For generation zero, only 60 mature worms were available to quantify survival times: 30 for the combined selection lines and 30 for the control line.

Heritability determinations

Control area worms that did not show any gonadal development were placed in 4-oz. (118 ml) plastic beakers (160 worms, 2 per beaker), containing a 1-cm layer of sediment and 2 cm of water (GF-C filtered Hudson River water) that was aereated continuously. Water was added regularly to maintain initial water levels. The beakers were sorted monthly and juveniles were transferred to separate beakers (up to 6 worms per dish and a maximum of 18 offspring per pair of adults). Some ground fishfood was added once the previously added food had disappeared. About half the pairs had produced offspring after 3–4 months. Resistance was again quantified as survival times in 8.9 μM Cd, 10.2 μM Ni, and 0.5 μM Co in water, using mature worms.

Heritability estimates were obtained using resemblances in survival times among relatives (Falconer, 1981), on log-transformed survival times. Estimates were obtained from the regressions of "mean offspring survival time" on "midparent survival time" (both on unweighted data, as well as the data weighted for the number of offspring per family), and "offspring survival time"

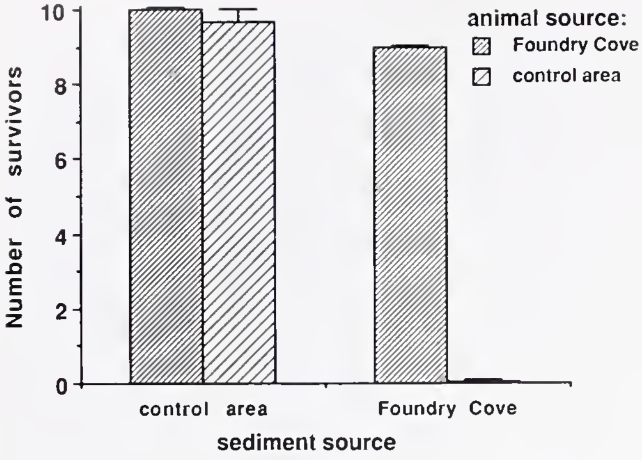

Figure 1. Numbers of *Limnodrilus hoffmeisteri* from Foundry Cove and the control area, surviving a 28-day exposure to sediments from each location. Mean and S.E.$_{mean}$ are shown, three replicates per group, each replicate started with 10 individuals.

on "parent survival time." The heritability (h^2) was computed as the regression coefficient (b) of the midparent-offspring regression, and $h^2 = 2b$ for the parent-offspring regression. The weighting for family size was performed according to Falconer (1981).

An additional estimate of the heritability was obtained from the results of the selection process. This "realized heritability" was determined as the cumulative response, divided by the cumulative selection differential (Falconer, 1981).

Results

Resistance comparisons

The first step in our investigation into the evolution of resistance to the metal-pollution of Foundry Cove was aimed at determining if *L. hoffmeisteri* from this area had an elevated resistance. These Foundry Cove worms were indeed more resistant to metal-rich sediment than their conspecifics from the control area (Fig. 1). The difference in survival was highly significant for animal source and animal × sediment interaction (both $P < 0.001$) in an ANOVA on the square root of the number of survivors.

An exposure to Cd, Ni, and Co in water revealed the presence of resistance differences among worms collected at sites with different metal levels in the sediment (Fig. 2). As before, worms from the routine collecting site in Foundry Cove (with about 7000 μg Cd per g sediment) were significantly more resistant than the ones from the control area [in unplanned comparisons among pairs of means using the Games and Howell method (Sokal and Rohlf, 1981) since variances were heterogeneous]. Within Foundry Cove, the animals from a relatively clean part of the cove were significantly less resistant than those from the routine collecting site. Though the resistance of worms from the most polluted site in Foundry Cove approached that of the worms from the routine collecting site, their resistance did not differ significantly from that of the worms from any of the other sites (probably due to the large variation in resistance among worms from the most polluted site).

As the individuals from Foundry Cove used in these experiments had previously encountered metal-rich sediment, the elevated resistance could have been due to physiological acclimation, genetic adaptation, or both. To investigate the presence of a genetic component in the resistance differences, resistance in the second generation offspring was compared to that of the worms from the control area and Foundry Cove. The results are shown in Figure 3. Overall, control area worms were significantly less resistant than both groups of Foundry Cove worms [in a multiple t-test (LSD) at $\alpha = 0.05$ on the square root of the number of survivors]. For the separate Cd levels, no statistically significant differences were evident at the low (430 μg/g Cd) and high (63,000 and 90,000 μg/g) Cd levels. Survival at 22,400 and 31,600 μg/g Cd showed the same pattern as for the overall data, while at 44,700 μg/g Cd the worms collected from Foundry Cove survived significantly better than the two other groups. These results demonstrated that *L. hoffmeisteri* from Foundry Cove was genetically adapted to the Cd-rich sediment. Overall, no significant difference in resistance was detected between the worms from

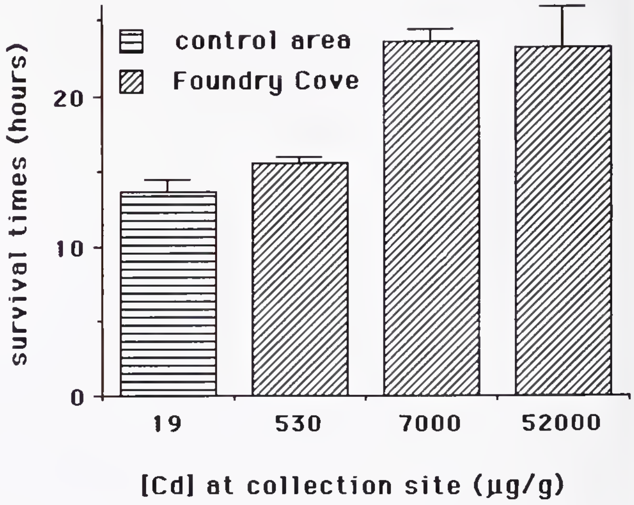

Figure 2. Survival times of *Limnodrilus hoffmeisteri* from the control area and three differently polluted sites in Foundry Cove, when exposed to a mixture of Cd, Ni, and Co in water (respect. 8.9, 10.2, and 0.5 μM). Mean and S.E.$_{mean}$ are shown, 7 replicates (with 6 worms each) per group.

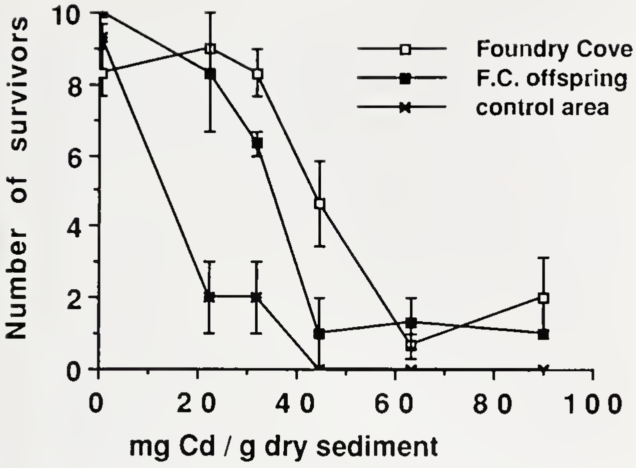

Figure 3. Numbers of field-collected *Limnodrilus hoffmeisteri* from Foundry Cove and the control area, and second generation offspring of Foundry Cove worms surviving a 28-day exposure to sediments with different Cd levels. Lines connect means, vertical lines represent $S.E._{\text{mean}}$. Three replicates per group, each replicate was started with 10 individuals.

Foundry Cove and their offspring that had never been exposed to metal-rich sediment.

Selection experiments

L. hoffmeisteri from the control area was selected for resistance to a combination of Cd, Ni, and Co in water, to determine the potential for the evolution of resistance in this population. No response to the selection was evident after one generation of selection but later generations showed a significantly higher resistance than the control line (Fig. 4). After three generations of selection, the difference in resistance between the selected lines and the control line averaged 66% of the difference in resistance between field-collected Foundry Cove worms and those from the nearby control site.

Heritability determinations

The potential for adaptation to metals in the control population was also assessed by determining the heritability of metal-resistance in this population. Regressions of the average resistance of offspring on the average resistance of their parents resulted in heritability estimates (±S.E.) of 0.93 ± 0.12 (n = 28) and 0.92 ± 0.12 (n = 28) (the latter value for data weighted for the number of offspring per family). Single parent-offspring regression resulted in an heritability estimate of 1.08 ± 0.10 (n = 455).

The result from the selection experiment translated into a realized heritability of 0.59 ± 0.14 (n = 11), somewhat lower than the heritability estimates given above, but still indicating the presence of much heritable genetic variation.

Discussion

Limnodrilus hoffmeisteri from Foundry Cove was much more resistant to both Foundry Cove sediment with high Cd, Ni, and Co levels and water spiked with these metals, than conspecifics from the control site. The elevated resistance remained after two generations in clean sediment. This demonstrated that *L. hoffmeisteri* from metal-polluted Foundry Cove was genetically adapted to the combination of cadmium, nickel, and cobalt. The second generation offspring (that had never encountered metal-rich sediment previously) were not less resistant than the worms that were collected in Foundry Cove. Therefore, we have no evidence for the presence of an environmental component in the resistance differences or for a decrease in resistance in the absence of a continued selection pressure. The occurrence of a genetic adaptation to metals in a population of oligochaetes inhabiting a metal polluted site agrees with findings for a population of isopods (Brown, 1976). While several other studies did not detect resistance differences among populations from differently polluted environments, this absence of resistance differences could be due to the possibility that those pollutants in question failed to affect these populations negatively (Klerks and Weis, 1987).

The metal adaptation in Foundry Cove worms was demonstrated about 30 years after the onset of the pollution at this site. This showed that metal resistance can

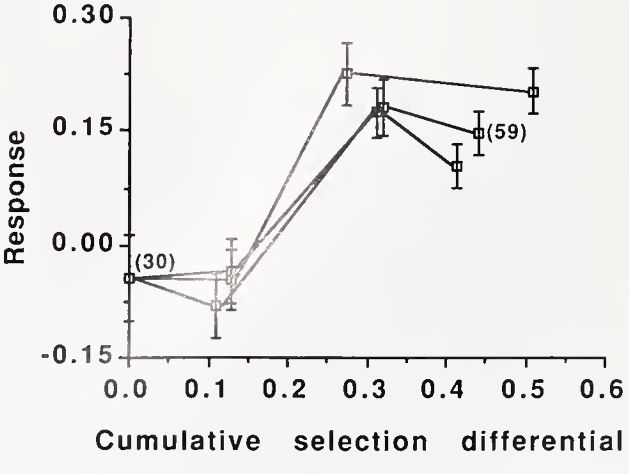

Figure 4. Selection response as a function of the cumulated selection differential, in *Limnodrilus hoffmeisteri* exposed to a mixture of Cd, Ni, and Co in water (respect. 8.9, 10.2, and 0.5 *μM*). Lines connect the response means in three selection lines (response determined as the difference in average survival time between a selected line and the control line), while vertical lines represent the 95% confidence interval of this difference). n = 60 for each determination, except where indicated otherwise.

evolve rapidly. Such an extremely rapid evolution of metal resistance was not evident from the metal adaptation in populations in S.W. England mentioned earlier (Bryan and Hummerstone, 1971; Brown, 1976), where the metal pollution has lasted more than several hundred years (ref. in Brown, 1976; Bryan and Gibbs, 1983).

We found a difference in metal resistance between Foundry Cove worms collected from sites (with different metal levels) that were only 200 m apart. This indicated that selection is possibly very strong. But the alternative, that gene-flow is extremely limited in these worms, cannot be ruled out since little is known about the amount of gene-flow in these animals. However, selection for metal resistance in natural populations can be strong enough to maintain resistance differences despite high gene flow (McNeilly, 1968).

We estimated that most of the variation in resistance in the control population is additive genetic variation and constitutes variation on which natural selection can act (h^2 values ranging from 0.6 to 1.0). It is possible that part of the resemblance in resistance between parents and offspring is due to the presence of a common environment (as the offspring spent up to 12 weeks in the dish containing their parents). This could have lead to an overestimation of the heritability. If the resemblance between parents and offspring was entirely due to this common environment, offspring should have resembled the individual parents to the same extent as the midparent. This was clearly not the case since the coefficient for the parent-offspring regression was 58% of the midparent-offspring regression (regression coefficients were 0.54 and 0.93, respectively). Therefore, it is unlikely that more than a minor proportion of the resemblances between parents and their offspring had an environmental basis.

The presence of much heritable variation in metal resistance has been reported for plants (McNeilly and Bradshaw, 1968), but no heritability estimates on populations of animals are available for comparison. The observed rapid evolution of resistance in *L. hoffmeisteri* is consistent with our heritability estimates. Using the conservative estimate for the heritability of 0.6, it takes (theoretically) 38 generations of selection at a fairly low intensity of selection (95% of the population contributing to the next generation), to obtain the resistance-difference observed between Foundry Cove and control area worms. With 40% of the population contributing to the next generation (which seems a realistic value for control area worms in Foundry Cove sediment based on our experiments) the resistance would have evolved in 4 generations, while at a high intensity of selection (¼% of the population contributing to the next generation), one generation of selection would have been sufficient.

The observed rapid evolution of resistance to metals is also consistent with the laboratory selection experiment. After three generations of selection, the response was equivalent to 66% of the resistance difference observed among the natural populations of *L. hoffmeisteri*. A positive response to selection for metal resistance was also obtained by Moraitou-Apostolopoulou *et al.* (1983) for a benthic copepod. In contrast, attempts to select for metal resistance were unsuccessful in flagfish (Rahel, 1981) and daphnids (LeBlanc, 1982).

The main importance of this study is not its demonstration of natural selection; natural selection in wild populations has been documented extensively (Endler, 1987). This study is unusual in that it demonstrated that the response to directional selection in natural populations can be extremely rapid. The spectacular potential of natural selection to change the physiological capabilities of aquatic invertebrate populations should also be realized in the context of predictions of the effects of pollutants on natural ecosystems. Evidence that populations may adapt to environmental pollutants is accumulating. However, it seems unlikely that all species will adapt to pollutants. For example, in the chironomid fly *Tanypus neopunctipennis* we found no difference in resistance between animals from Foundry Cove and the control site. This may relate to the greater gene flow among sites in this species, or the lower metal accumulation (Klerks, 1987). A number of species are missing from the most polluted site in Foundry Cove, similar to the situation in many polluted environments (Klerks and Weis, 1987). Therefore it would be totally unjustified to see the evolution of resistance as a justification for the release of pollutants in natural environments.

Acknowledgments

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Abundance in Bottom Sediments and Hatching Requirements of Eggs of *Centropages hamatus* (Copepoda: Calanoida) From the Alligator Harbor Region, Florida

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Abstract. The distribution and abundance of *Centropages hamatus* eggs in the upper 5 cm of the sea-bottom of the Alligator Harbor region, Florida, was determined. The hatching of these eggs at 4, 10, 15, 19, and room temperature (20–25°C) was monitored in the laboratory. Hatching only occurred at temperatures less than 20°C. Eggs produced by *C. hamatus* individuals that were reared in the laboratory at 19°C, 20L–4D did not hatch at 19°C. A 60% hatch was induced by incubating these eggs at room temperature (22–25°C) for 60 days followed by cooling at 15°C. The results indicate that *C. hamatus* produces diapause eggs that are capable of surviving warm temperatures characteristic of late spring and summer months. Since planktonic phases of this species are not present in the water column at temperatures greater than 20°C, it appears that diapause is a critical life history stage without which the life cycle would be broken.

Introduction

In temperate-boreal waters many planktonic copepod species undergo marked fluctuations in seasonal abundance to the extent that some species disappear entirely from the water column for portions of the year. The renewed development of benthic diapause eggs plays a fundamental role in the perpetuation of such species year after year in these regions (as reviewed by Grice and Marcus, 1981; Uye, 1985). However, the widespread geographic significance of diapause eggs is not clear since all studies have focused on temperate systems. Studies of copepod population dynamics in some sub-tropical and tropical waters have led investigators to suggest that resting eggs occur in these areas as well (Tranter and Abraham, 1971; Reeve, 1975; Fleminger, 1979), but the actual existence of such eggs has not been demonstrated. Moreover, it is not clear if such eggs would be subitaneous eggs whose development had been delayed temporarily due to unfavorable conditions in the sediment (*e.g.*, low oxygen) or diapause eggs. Unlike subitaneous eggs, which are capable of developing without any delay, diapause eggs must first complete a refractory phase during which time they will not hatch even if conditions are suitable. Hatching can only occur once this phase is completed and then only if conditions (*e.g.*, temperature, salinity, oxygen) are suitable. The subitaneous and diapause eggs of some copepod species can be clearly distinguished on the basis of external morphology (*e.g.*, the presence or absence of spines), but others are not morphologically distinct and can only be classified by determining the conditions that are necessary for hatching to occur (as reviewed by Grice and Marcus, 1981).

A preliminary analysis of bottom sediments from the Alligator Harbor region, Florida, in the late spring and summer 1988 (Marcus, unpub.) revealed the existence of benthic resting eggs of several planktonic copepod species: *Labidocera aestiva, L. scotti, Acartia tonsa, Centropages furcatus,* and *C. hamatus.* Species identifications were made by comparing the morphology of eggs (*e.g.*, diameter, surface ornamentation, color) isolated from the sediments to the morphology of eggs produced by females that were maintained in the laboratory, and by rearing nauplii that hatched from the eggs to late-copepodite and adult stages which could be unequivocally identified. Since adults of *C. hamatus* were not present

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in the water column at the time of the sediment collections, the species was chosen for further study, because the absence of planktonic stages in this northern subtropical embayment suggested that the eggs that were found were diapause eggs. These eggs were spherical, approximately 72 μm in diameter, and covered with spines that were approximately 18 μm in length.

Centropages hamatus (Lilljeborg) is a geographically widespread species. Along the coast of North America it has been reported from the Straits of Belle Isle (Pinhey, 1926) to Alligator Harbor in the Gulf of Mexico (Grice, 1956). In Alligator Harbor, the species has been described as a characteristic member of the winter copepod population occurring in the water column from November to April (Grice, 1956). In areas along the Atlantic coast of the United States south of Cape Cod, Massachusetts, the species has been reported to occur seasonally, typically during the winter and spring (see Deevey, 1960). Occasionally, however, when summer temperatures were abnormally low in these areas, it occurred throughout the year, *e.g.*, in Delaware Bay (Deevey, 1960). In the Gulf of Maine (Bigelow, 1926) and on Georges Bank (Davis, 1987), the species has been reported to occur during the late summer and fall.

C. hamatus has been reported to produce two morphological types of eggs in the White Sea (Pertzova, 1974) and it was later suggested (Grice and Marcus, 1981) that the eggs produced in the fall were diapause eggs. Other evidence for the existence of resting eggs was the appearance of *C. hamatus* nauplii following the laboratory-incubation of bottom sediments that were collected from Buzzards Bay, Massachusetts, Georges Bank, the English Channel, and the southern North Sea (Marcus, 1984, unpubl.; Lindley, 1986). However, it is not clear whether the nauplii that appeared were derived from diapause eggs or subitaneous eggs that were delayed in development due to adverse conditions in the sediments.

This study was conducted to determine the distribution and abundance of eggs of *C. hamatus* in the bottom sediments of the Alligator Harbor region. The hatching requirements of these eggs, and eggs obtained from animals that were reared in the laboratory, were ascertained. The results show that *C. hamatus* does produce diapause eggs. It is suggested that these eggs enable the species to survive warm summer temperatures.

Materials and Methods

Determination of egg distribution in the sediments

Sampling was conducted from July 9, 1988, through October 20, 1988, at six stations in the Alligator Harbor region, Florida (Fig. 1). At each station sediments were collected with a hand-held pole corer (Lively, unpubl.), water depth was recorded from a meter scale on the pole, and surface temperature and salinity were determined with a Y.S.I. Model 33 S-C-T Meter. With the pole corer, cylindrical core tubes (4.8 × 30 cm D × L) were slowly pushed into the sea-bottom to a depth of about 15 cm. A ball valve was then triggered to close, trapping the sediment, and the unit was hauled to the surface. Cores were kept in an insulated container from the time of collection until they were returned to the laboratory. For some collections, the cores were additionally packed in ice. In the laboratory, cores were divided at intervals as fine as 2 mm and as coarse as 1 cm using a core extruder (Fuller and Butman, 1988). The material in each layer was suspended in 5 μm filtered seawater, sonicated for 30 s (#4 setting, Branson Sonifier Cell Disruptor 200), filtered through a 48 μm Nitex screen, suspended in a concentrated solution of table sugar (1:1 sugar:distilled water), and centrifuged for 3 min. The material remaining in suspension was filtered through a 48 μm screen, washed thoroughly with seawater, and transferred to a dish containing seawater. The final fraction was examined under a dissecting microscope and eggs of *C. hamatus* were counted and transferred to small wells containing seawater. The eggs were kept at room temperature (22–25°C) and monitored for hatching.

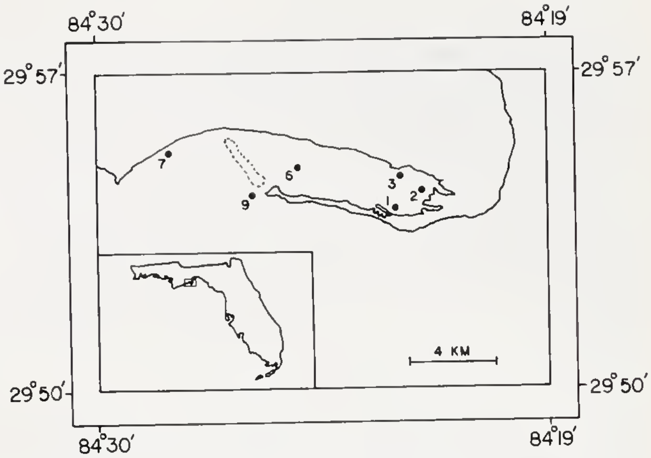

Figure 1. Location of six sampling stations in the Alligator Harbor region, Florida.

Qualitative plankton samples were also obtained from surface waters throughout the study. A 153 μm mesh net was towed for 2 to 5 min and the collected material was returned to the laboratory in insulated containers. Samples were examined under a dissecting microscope to determine if *C. hamatus* adults were present.

Determination of hatching requirements

Experiment 1 was conducted with eggs collected on July 9. The sediment samples were held at 4 ± 1°C for 2

Table I

Mean number (×10^4) and standard deviation (S.D.) of eggs m^{-2} of Centropages hamatus *in the top 5 cm of bottom sediments from the Alligator Harbor region, Florida, at each station for the period from July 9 through October 20, 1988*

Station	1	2	3	6	7	9
Water depth (m)	1.6	1.6	1.6	2.1	3.5	4.0
# of cores	3	2	2	2	10	3
Mean	1.8	0.4	0.0	0.7	7.3	3.6
S.D.	0.9	0.2	0.0	0.5	6.2	2.1

to 4 days prior to isolation of the eggs. The eggs were then held at room temperature (22–25°C) for 5 to 7 days. During this time no eggs hatched and the eggs were subsequently distributed into five jars (10 eggs/jar). Two jars were placed at 4°C, and three were held at room temperature (22–25°C). After 59 days the jars at 22–25°C were transferred to 10, 15, and 19 ± 1°C since none of the eggs had hatched.

Experiment 2 was conducted with a mixture of eggs from two collection dates, July 9 and July 26. In this case the sediment samples were held at 4°C for as much as 11 days prior to isolation of the eggs. The initial incubation period at room temperature (22–25°C) ranged from 1 to 11 days. As in Experiment 1, no eggs hatched during the warm period so 119 eggs were placed in a jar and stored at 4°C.

Experiments 3 and 4 were conducted with eggs collected on September 14 and October 20, respectively. Eggs were not exposed to an initial period at 4°C prior to their isolation, nor a pre-incubation at room temperature. Rather, as soon as the eggs were isolated from the samples they were distributed into jars and placed at either 4, 10, 15, 19, or room temperature (20–24°C). In Experiment 3 each jar contained 15 eggs, but there were not enough eggs to have replicates at each temperature. In Experiment 4 each temperature condition was tested in duplicate with 10 eggs/jar.

Hatching was monitored periodically. Some of the nauplii that hatched were reared to reproductive maturity on a mixed diet of 4 dinoflagellates (*Gymnodinium nelsoni, Gonyaulax polyedra, Prorocentrum micans,* and *Scrippsiella trochoidea*) at 19°C and 20L–4D (h light–h dark). The eggs produced by these animals were kept in a dish of seawater at 19°C for 3 days. None of the eggs hatched so they were distributed into jars (15 eggs/jar) which were then held at room temperature (22–25°C) for periods ranging from 2 to 60 days. Each jar was transferred to 15°C following its particular incubation interval at room temperature. Hatching at the cooler temperature was monitored daily.

Results

Large numbers of eggs of *C. hamatus* were found in the sediments of the Alligator Harbor region at water depths ranging from 1.6 to 4.0 m (Table I). The eggs occurred as deep as 5 cm (the maximum depth analyzed in this study). The average abundance of eggs in the top 5 cm ranged from 0 to 7.3 × 10^4 eggs m^{-2} (Range: 0 to 2.1 × 10^5). Greater densities of eggs were found in the deeper waters outside of Alligator Harbor at Stations 7 and 9. Eggs were found from July through October and during this time adults of *C. hamatus* were not present in the water column. During July and August, surface water temperature and salinity ranged from 26.0 to 28.5°C and 32 to 35‰, respectively. By October, water temperature and salinity had declined to 18.0 to 20.0°C, and 27 to 31‰, respectively.

Eggs collected in July (Experiments 1 and 2) did not hatch at 22–25°C even after 2 months of incubation. Hatching occured at each of the other incubation temperatures, and the time to hatching was inversely related to temperature (Fig. 2). Eggs collected in September and October (Experiments 3 and 4) hatched at each of the incubation conditions. The cumulative hatch for the eggs held at 4, 10, 15, and 19°C are shown in Figure 2. As in Experiments 1 and 2 hatching was most rapid at the warmer temperatures. In Experiments 3 and 4 the cumulative hatch of eggs held at room temperature (20–24°C) was 33% and 90%, respectively. In these experiments hatching occurred in the jars at room temperature only after the temperature had dropped to 20°C.

Eggs that were produced by the laboratory-reared animals hatched at 15°C, but only after prolonged exposure to warm room temperatures (22–25°C). The first hatch (*i.e.*, one nauplius) occurred after 12 days at 15°C, in the

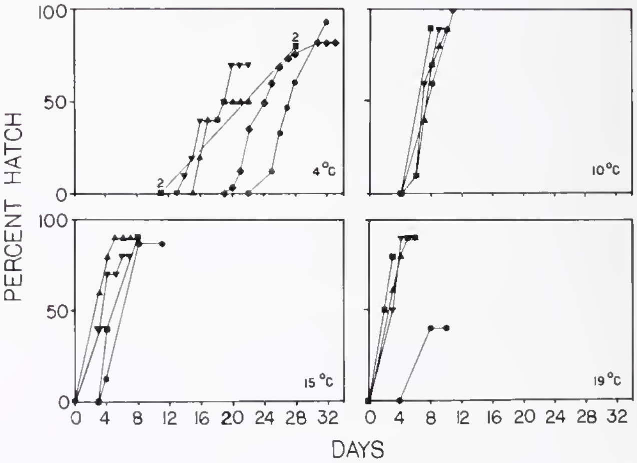

Figure 2. Cumulative percent hatch of *Centropages hamatus* eggs collected from bottom sediments and incubated in the laboratory. Experiments 1 (■), 2 (◆), 3 (●), and 4 (▲,▼) duplicates).

jar kept at room temperature for 38 days. A greater hatch (60%) occurred in the jar held at room temperature for 60 days. Hatching began after four days at 15°C and continued for 44 days.

Discussion

The abundance of eggs of *C. hamatus* in the sediments of the Alligator Harbor region, Florida was high. The greatest density, $2.1 \times 10^5\ m^{-2}$, is of the same order of magnitude as that reported for eggs of *Labidocera aestiva* in Buzzards Bay, Massachusetts (Marcus and Fuller, in press). Higher numbers tended to occur in deeper water outside the embayment. The fact that these eggs hatched upon incubation in the laboratory indicates that eggs of *C. hamatus* can survive for as long as 5 months in bottom sediments. This is a minimum estimate and assumes that the eggs collected in October were produced in April just prior to the disappearance of the species from the water column. In the Alligator Harbor region, adults of *C. hamatus* occur from November through April (Grice, 1956). During the time that *C. hamatus* is absent from the water column temperatures typically range from approximately 20 to 32°C (Grice, 1956). Since the results of this study indicate that eggs only hatch at temperatures equal to or less than 20°C, one might conclude that eggs are simply inhibited from hatching during the late-spring and summer periods due to warm temperatures. When the water temperature drops below 20°C in October, the eggs hatch and give rise to the adults that appear in November.

The hatching response of eggs that were produced by animals reared at 19°C, 20L–4D indicates that *C. hamatus* has the potential to produce diapause eggs (according to the criteria of Grice and Marcus, 1981). None of the eggs produced by laboratory-reared animals hatched at 19°C initially, but some of these eggs did hatch at 15°C after prolonged incubation at warm room temperatures (22–25°C). Although, 20L–4D constitutes an abnormally long daylength never experienced by *C. hamatus*, the results demonstrate that the species has the biological capacity to produce diapause eggs. In a previous study (Marcus, unpubl.), *C. hamatus* from Woods Hole, Massachusetts, were reared at 19°C, 12L–12D and produced thousands of eggs. Many of these eggs hatched within a few days at 19°C, but many did not hatch. The precise percentage of eggs that hatched was not determined. Some of the unhatched eggs were placed in jars containing filtered seawater, stored at room temperature for approximately 3 months, and then transferred to 5°C. No hatch occurred while the eggs were held at room temperature, but after the eggs were transferred to 5°C some hatch occurred after 13 days with large numbers of nauplii noted after 17 days. Although a quantitative record was not kept, the results are also indicative of diapause. Since the eggs produced by Woods Hole animals were kept for 3 months at room temperature it may be that the eggs of the laboratory-reared *C. hamatus* from Florida were not incubated sufficiently long to yield a rapid and synchronous hatch at cool temperatures (*i.e.*, 15°C). Unlike the report of Pertzova (1974) on *C. hamatus* from the White Sea, only one morphological type of egg was observed for *C. hamatus* from Woods Hole and Florida. It was spherical and uniformly covered with spines.

Only two other studies have reported on the hatching requirements of resting eggs produced by winter-spring marine copepods. Sullivan and McManus (1986) showed that some eggs produced by *Acartia hudsonica* from Narragansett Bay, Rhode Island only hatched at cold temperatures *i.e.*, 5°C. A requirement for an intervening period of warm temperatures was not demonstrated. However, the eggs of *A. clausi* from Japanese waters appear to require a period of warm temperatures followed by chilling to induce hatching (Uye, 1985).

Marcus (1979) showed that the refractory phase (*i.e.*, the period of time during which diapause eggs will not hatch even if environmental conditions are favorable) of eggs of *L. aestiva* was shortened by chilling at 5°C, but that diapause eggs would eventually hatch after several months at warm temperatures even if they had not been chilled. The difference was that pre-chilled eggs hatched synchronously. It is not clear if the opposite condition holds for *C. hamatus; i.e.*, would eggs hatch at temperatures less than about 20°C if they were not exposed to a period of warm temperatures? The temperature conditions that are required for embryonic development and hatching must be taken into account to predict the contribution of benthic diapause eggs to the population growth of species. For example, along the northeast coast of the United States it is likely that diapause eggs residing on the sea-bottom rarely experience temperatures above 20°C. If exposure to temperatures warmer than 20°C is required for hatching, then these eggs should not be considered as a source of potential recruits to the planktonic population. On the other hand, if such warm temperatures are not necessary, then such eggs represent an important potential source of nauplii for growth of the planktonic population. Since the annual environmental cycles in waters of the western north Atlantic are different than the conditions in the northeastern Gulf of Mexico, it is likely that the precise conditions that are necessary for the induction, maintainence, and termination of diapause are different. Regardless of these geographic differences, in areas where *C. hamatus* disappears entirely from the water column for portions of the year, diapause eggs enable survival of individuals during warm seasons and must be essential for the perpetuation of species year after year.

Acknowledgments

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The Effect of *Hydra* on the Outcome of Competition Between *Daphnia* and *Simocephalus*

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Abstract. The cladoceran genera *Daphnia* and *Simocephalus* often co-occur in nature. In laboratory experiments, populations of the two genera had similar growth rates when grown separately, but when cultured together *Daphnia* invariably excluded *Simocephalus*. However, the added presence of the littoral zone predator, *Hydra*, reversed this trend with *Simocephalus* remaining after *Daphnia* had been eliminated. This result was robust in culture vessels as small as 100 ml and as large as 85 l. It is hypothesized that *Simocephalus* has evolved a suite of energetically expensive traits to deter littoral zone predators, whereas *Daphnia*, which are planktonic, have not evolved such costly traits and hence have more energy available for reproduction and are able to exclude *Simocephalus*.

Introduction

Over evolutionary time prey species have limited the impact of predation by behavioral, physiological, and morphological adaptations (Sih, 1987). The consequences of these adaptations are not restricted to the predator-prey relationship, but may also be important in other facets of the ecology of the prey species. For example, morphological features of many planktonic cladocerans not only deter predation, but also effect competitive interactions. Predator-resistant morphs of *Bosmina* and *Ceriodaphnia* are competitively inferior to predator susceptible morphs (Nilssen *et al.*, 1980; Zaret, 1972a; b). The maintenance of such morphological diversity has been termed predator balanced polymorphism (Zaret, 1969). The energetic cost of predator deterrence is apparently reflected in reduced fecundity and consequently reduced competitive ability (Kerfoot, 1975, 1977; Jacobs, 1978; O'Brien and Vinyard, 1978; Cooper and Smith, 1982). When predator pressure is low, the susceptible morph is able to numerically dominate the protected morph. While the maintenance of distinct morphs is striking, predation has also been a significant selective force in the evolution of previously overlooked characteristics which not only reduce predation rates, but which also effect interactions between prey species.

Schwartz *et al.* (1983) demonstrated that *Hydra* are efficient predators of the cladoceran *Daphnia*, but rarely capture members of the closely related genus, *Simocephalus*, which inhabit the littoral zone along with *Hydra*. *Simocephalus* has a suite of characteristics that deter predation by *Hydra*. The evolutionary origin of each trait is unknown, but may be, in part, the result of selective pressure by *Hydra* or other littoral zone predators. The behavior of *Simocephalus* is such that it spends most of the time attached to substrates and is therefore not in the water column where it might encounter the tentacles of *Hydra*. In addition, physiological adaptations allow *Simocephalus* to swim among the tentacles of *Hydra* without eliciting the discharge of nematocysts. Finally, when nematocysts are discharged, *Simocephalus* is unharmed, suggesting that the nematocysts fail to penetrate this cladoceran's heavy carapace. In contrast, *Daphnia*, which is planktonic, is highly vulnerable to predation by *Hydra*. One explanation for the vulnerability of *Daphnia* is that, because *Daphnia* rarely encounters *Hydra*, it has failed to evolve mechanisms that reduce the impact of the predator.

Is there a cost to *Simocephalus* associated with its suite of anti-predator adaptations? Competition experiments between *Daphnia* and *Simocephalus* have invariably shown that *Daphnia* outcompetes its relative (Frank, 1952; Corigliano and de Bernardi, 1978; de Bernardi and

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Manca, 1981; 1982; Crowley and Johnson, 1983). In general, these studies indicate that *Simocephalus* has a lower intrinsic rate of increase than *Daphnia* and this rate is additionally reduced by the presence of the competitor. The results of these competition experiments have been explained by proximate differences in life history characteristics, without attempting to examine the ultimate factors that have selected these suites of traits. The role of predation has, for instance, been neglected in explanations of the relative position of the two genera in a competitive hierarchy.

In this paper we show that the outcome of laboratory competition experiments between *Daphnia* and *Simocephalus* can be altered by the presence of a predator that consistently selects the prey species that has not been exposed to selective pressures favoring the evolution of appropriate defensive tactics. We suggest that the reduced competitive ability of *Simocephalus* as compared to *Daphnia* is largely the result of energetically expensive anti-predator adaptations required for life in the littoral zone.

Materials and Methods

The effect of *Hydra oligactis* predation on the outcome of competition between *Daphnia pulex* and *Simocephalus vetulus,* two commonly co-occurring cladocerans, was evaluated in three sets of laboratory experiments. Both cladoceran species and the *Hydra* were clonal populations (*Daphnia pulex* clone W1-1) maintained in the laboratory and originally collected in the Windsor, Ontario, area. Experiments were initiated in synthetic pond water (Hebert and Crease, 1980), but this was gradually replaced during the experiments with conditioned tap water containing algal food. All experiments were conducted at 15°C and 12:12 LD. During the experiments the cladocerans were fed at two-day intervals with a mixed algal culture, primarily *Scenedesmus* sp., maintained in the laboratory. No attempt was made to quantify the feeding regime as all experiments were conducted concurrently; *i.e.*, all vessels in an experiment received the same amount and quality of food. As control populations showed a rapid numerical increase (Figs. 1, 4), it was assumed that the feeding regime was adequate.

The first two experiments were similar in design, differing primarily in the size of the vessel used. In one set of experiments, 120 ml plastic cups were filled to 100 ml. Each treatment was replicated in triplicate. Ovigerous females were used to initiate each replicate cup. Control cups received six individuals from one of the two cladoceran species. Competition cups were initiated with three individuals from each species. Additional competition cups were also set up with six *D. pulex* and three *S. vetulus* to determine if *Daphnia* would more quickly outcompete *Simocephalus* under these conditions. The impact of a selective predator, *Hydra,* was determined in another set of competition cups (initiated with three females from each species) as well as control cups (with six individuals of each species), with each cup receiving one adult *Hydra,* without buds. All individuals were counted in each cup once a week for five weeks.

The impact of container size was examined in a second set of experiments conducted in 200-ml finger bowls. The experimental design was similar to the first except that the treatments with six *Daphnia* and three *Simocephalus* and the individual species with *Hydra* were eliminated. All treatments were run in triplicate. Again, all individuals in each finger bowl were enumerated weekly during the seven week duration of the experiment.

Data were analyzed using an analysis of variance which took into account the experimental design of three factors (experiment, species, and weeks) with repeated measures on only one of the factors (weeks) (Winer, 1962). The large differences between initial and final population size from each of the preceding experiments necessitated the use of log $(x + 1)$ transformed data for statistical analysis. The experiment initiated with twice as many *Daphnia* as *Simocephalus* was not included in this analysis.

A final competition experiment was conducted in 100-l aquaria filled with 85 l of synthetic pond water. There were two control and two treatment aquaria. All aquaria were initiated with 20 individuals each of *D. pulex* and *S. vetulus.* In addition, the experimental aquaria also received a single *Hydra.* The aquaria were checked at two-day intervals and maintained at 85 l with the same algal mixture used in the other experiments. This experiment was allowed to run for six weeks at which time the contents of the aquaria were filtered through a plankton net and preserved in ethanol. Due to the large number of individuals present in the aquaria at this time, the individuals from three subsamples were sorted to species, counted, and then dried at 80°C for 24 h and weighed. The remainder of each sample was then dried and weighed in the same manner and the total number of individuals belonging to each species estimated on the basis of its abundance in the original subsamples.

Results

The results of the small cup and finger bowl experiments were similar, differing primarily in their final population size. The finger bowls supported about 50% more individuals of both cladoceran species (compare Figs. 1 and 4). The rapid numerical increase in all vessels indi-

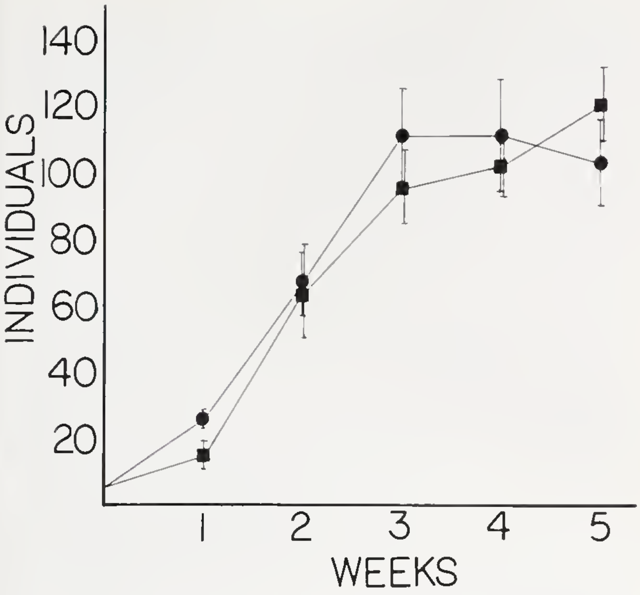

Figure 1. The mean population size of *Daphnia pulex* (circles) and *Simocephalus vetulus* (squares) when grown separately in 100 ml cups during a 5-week period. Bars represent ±2 S.E.

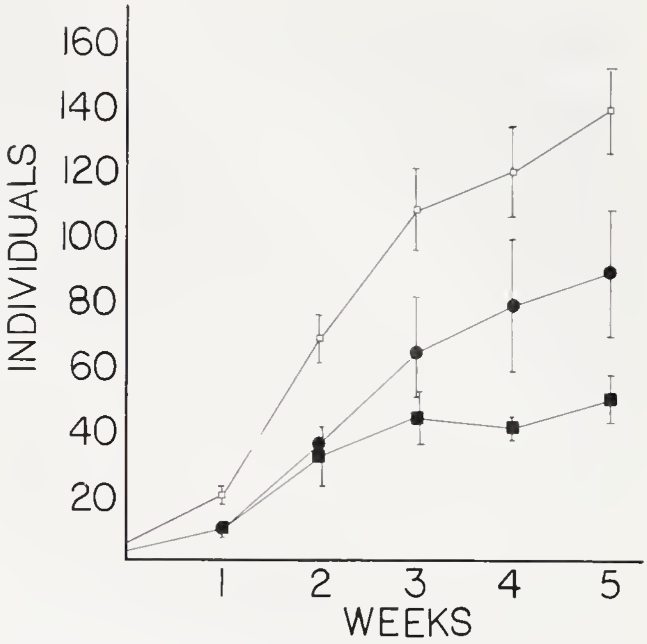

Figure 2. The mean population size of *Daphnia pulex* (darkened circles) and *Simocephalus vetulus* (darkened squares) when grown together in 100 ml cups during a 5-week period. Open squares represent the mean total cladoceran population in the cups. Bars represent ±2 S.E.

cates the adequacy of the feeding regime. Visual inspection of the untransformed data (Figs. 1, 2, 4, 5) suggested that the growth of all test populations could be divided into two distinct phases. A period of rapid, linear, population growth was followed by a period during which the populations leveled off, apparently having reached their "carrying capacity." The initial phase lasted three weeks in the cups and four weeks in the finger bowls.

The analysis of variance (Tables I, II) indicated that in both sets of experiments there was a significant difference between the population size in the control and experimental vessels. This may be due to the fact that the trajectory of total population growth was slower in the experimental vessels (compare Figs. 1, 2 and 3, 4). However, the combined carrying capacity in all of the competition vessels was greater than the population in the control vessels, which may indicate that the two species utilized slightly different food resources.

There was a significant difference in population size between the species only in the bowls (Table II). Thus, in the bowls at least, there were significantly fewer *Simocephalus* than *Daphnia* in the experimental vessels than

Table I

ANOVA for the 100-ml cup set of experiments

Source	df	SS	MS	F
Between cups	9	1.49		
A (experiment)	1	1.06	1.06	28.91**
B (species)	1	0.16	0.16	4.36
AB	1	0.05	0.05	1.47
Error	6	0.22	0.04	
Within cups	50	14.15		
C (weeks)	5	11.94	2.39	35.16**
AC	5	0.02	0.00	0.06
BC	5	0.06	0.01	0.20
ABC	5	0.10	0.02	0.28
Error	30	2.04	0.07	

Table II

ANOVA for 200-ml finger bowl experiments

Source	df	SS	MS	F
Between bowls	10	2.93		
A (experiments)	1	2.24	2.24	60.98**
B (species)	1	0.26	0.26	6.97*
AB	1	0.18	0.18	4.88
Error	7	0.26	0.04	
Within bowls	77	20.79		
C (weeks)	6	19.61	3.27	400.94**
AC	6	0.04	0.01	0.86
BC	6	0.94	0.16	19.14**
ABC	6	0.00	0.00	0.00
Error	53	0.43	0.01	

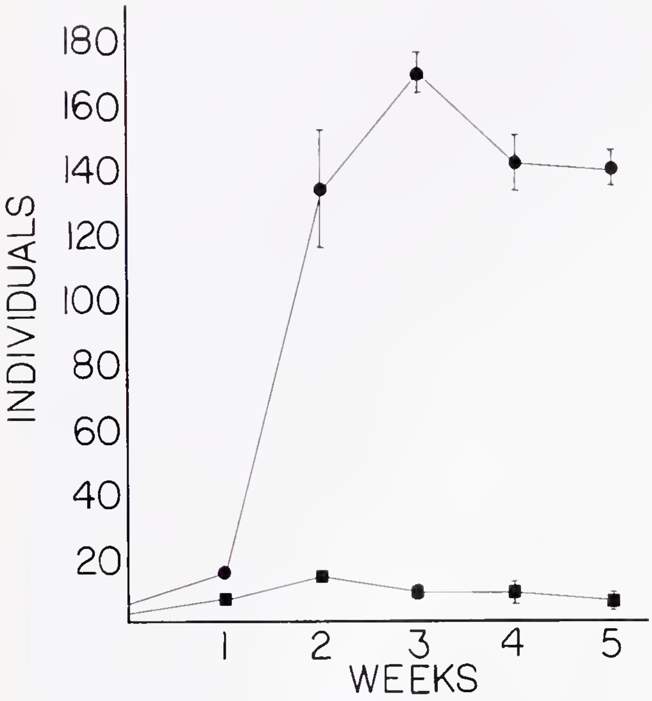

Figure 3. The mean population size of *Daphnia pulex* (circles) and *Simocephalus vetulus* (squares) from an initial population containing twice as many individuals of *Daphnia* as *Simocephalus*. The animals were grown in 100-ml cups for a 5-week period. Bars represent ±2 S.E.

in the control vessels. In both sets of experiments there was a significant difference associated with time, *i.e.*, not surprisingly the populations were significantly larger at the end of the observations than at the beginning.

The only interaction term found to be significant was the species by vessel interaction in the bowl experiments, *i.e.*, the species were changing in a different manner through time. Most likely the *Daphnia* population grew faster in the experimental bowls than the *Simocephalus* population through a suppression of the latter by either exploitative or interference competition.

Competitive replacement did not occur in any of the competition vessels, including the cups initiated with six *Daphnia* and three *Simocephalus* (Fig. 3). After only two weeks, *Daphnia* made up more than 90% of these populations. By the end of five weeks, *Simocephalus* populations had decreased to less than 5% of the *Daphnia* population. De Bernardi and Manca (1982) similarly found that competitive replacement is a slow process with *Simocephalus* populations persisting at low densities over a long period.

Those vessels which were initiated with *Hydra* in addition to the cladocerans all showed the same pattern: *Daphnia* individuals were eliminated in 97% of the vessels within the first week and, in all cases, by the end of three weeks. *Simocephalus* achieved almost 40% of the densities observed in the control population in the cups until the fifth week when *Hydra*, which had been unable to reproduce due to the low densities of suitable prey, died. *Simocephalus* then rapidly increased to 76% of the size of the control populations. Similarly, in the finger bowls, *Simocephalus* reached 82% of densities in the control by the end of the seventh week (Fig. 4).

Similar results were observed in the aquarium study indicating that the outcome was robust over a range of container size (Fig. 6). In the aquaria lacking predators, *Daphnia* outnumbered *Simocephalus* individuals although the two aquaria differed in degree of dominance, the ratio being 1.4:1 in one aquarium and 7.1:1 in the other. By contrast, all *Daphnia* individuals were eliminated in the aquaria with *Hydra*. Because only one *Hydra* was used to initiate the experiment, *Daphnia* populations expanded before the *Hydra* reproduced sufficiently to make a significant impact on their density. However, the large *Daphnia* population then served as food for the rapidly increasing predator population. By the end of six weeks, all *Daphnia* individuals had been eliminated leav-

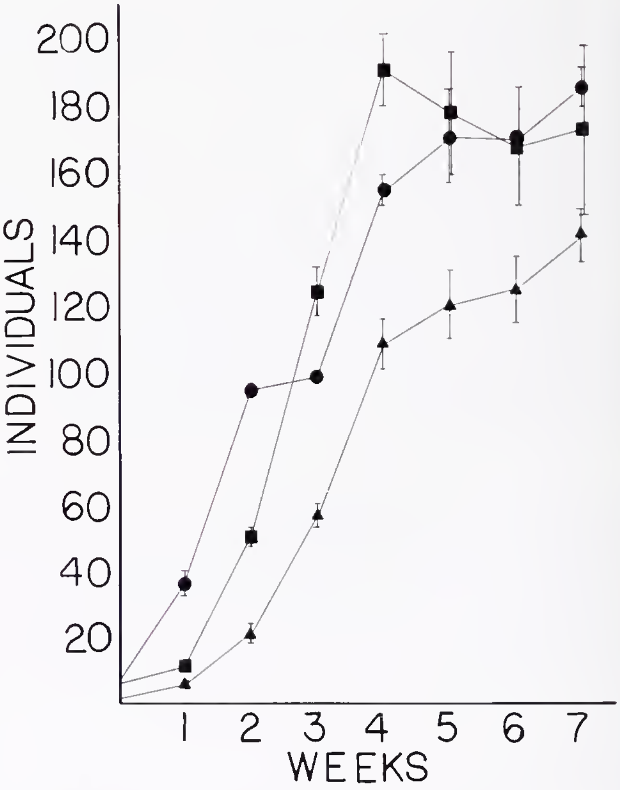

Figure 4. The mean population size of *Daphnia pulex* (circles) and *Simocephalus vetulus* (squares) when grown separately in 200-ml finger bowls during a 7-week period. The mean population size of *S. vetulus* in the presence of *Hydra* is indicated by triangles. Bars represent ±2 S.E.

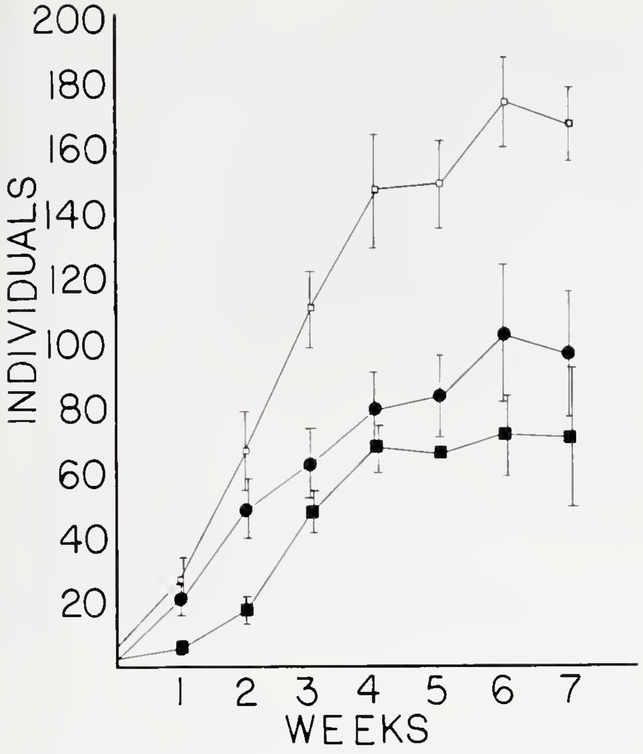

Figure 5. The mean population size of *Daphnia pulex* (circles) and *Simocephalus vetulus* (squares) when grown together in 200-ml finger bowls during a 7-week period. Open squares represent the total cladoceran population. Bars represent ±2 S.E.

ing an average of 9625 *Simocephalus* and 853 *Hydra*. There were many more *Simocephalus* in the experimental aquaria than total cladocerans in the control aquaria.

Discussion

It has often been demonstrated that predators may determine the outcome of competition between two prey species (Kerfoot, 1975, 1977; Jacobs, 1978; O'Brien and Vinyard, 1978; Cooper and Smith, 1982). In the present study the rapidity of the predators' effect depended on container size and was observed within a few days or at most a few weeks. As observed in previous studies, *Daphnia* outcompeted *Simocephalus* in the absence of predators. This latter result is in part due to *Simocephalus* reducing its reproductive rate in the presence of *Daphnia*, but of greater importance is the higher intrinsic reproductive rate of *Daphnia*. However, in the presence of predators the outcome of competition was determined by the evolutionary interaction between *Simocephalus* and these predators. *Daphnia* and *Simocephalus* have faced different predation regimes over evolutionary time and have responded to these differing selective pressures with unique suites of adaptations to limit the impact of predation. The cost of these adaptations is, in part, responsible for the differing competitive ability of the two genera.

Simocephalus is a genus that occupies the littoral zone where many of the predators employ ambush tactics (Greene, 1985). Faced with such predators one successful prey strategy is to avoid ambush by not swimming. Consequently, the sedentary behavior of *Simocephalus* can be viewed as a means of avoiding predation. *Simocephalus* makes use of glands on the dorsal portion of the carapace which allow it to attach to substrates and continue filter feeding. This behavioral adaptation frees *Simocephalus* of certain constraints on morphology; *i.e.*, the cost of developing a more robust exoskeleton is derived solely from costs of producing the structure, whereas planktonic cladocerans incur additional costs associated with increased weight.

Physiological adaptations similar to that which allows *Simocephalus* not to elicit the firing of coelenterate nematocysts have evolved many times in the Crustacea. Cyprid larvae of the barnacle *Balanus crenatus* do not elicit the discharge of nematocysts nor the mouth opening response of *Obelia dichotoma* (Standing, 1976) although larvae that are damaged are consumed. Numerous copepod genera live in association with sea anemones (Gotto, 1979; Humes, 1982). A well-studied example

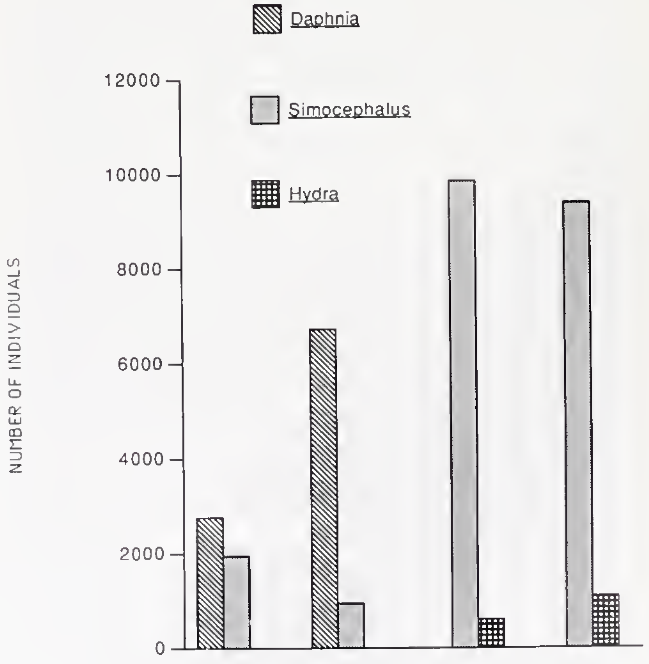

Figure 6. A comparison of the cladoceran populations in 100-l aquaria after 6 weeks. The two pairs of columns on the left represent the two control aquaria and the other two pair represent aquaria to which *Hydra* were also added.

is *Paranthessius anemoniae*, which moves freely over the tentacles of *Anemonia sulcata* (Briggs, 1976). However, free-living copepod species are paralyzed and consumed. In addition, an extract of the anemone's tentacles caused paralysis among the free-living copepods but not in *P. anemoniae*. Similar results have been reported for the lichomolgid copepod, *Doridicola*, which lives on a number of species of anemones and does not elicit the firing of nematocysts (Lonning and Vader, 1984). There are also amphipods which are associated with sea anemones that are immune to its host's toxins (Vader and Lonning, 1973).

The immunity to coelenterate nematocysts has also been suggested to be important in determining the outcome of competition between two hermit crab genera, *Pagurus* and *Clibanarius* (Wright, 1973). The former genus is able to occupy shells to which hydroids are attached, while the latter genus is stung and therefore cannot inhabit these shells. Thus the adaptations of *Simocephalus* are similar to those found in a number of other crustaceans. A likely explanation is that *Simocephalus* has evolved biochemical products that mask its presence, as the first step in the firing of nematocysts by *Hydra* is chemosensory (Lenhoff, 1968). Once this mask is breached, *Hydra* readily consumes *Simocephalus* (Schwartz *et al.*, 1983). The energetic cost of such an adaptation has not been determined, but is most likely small in comparison with the benefits.

In contrast to *Simocephalus*, *Daphnia* is unable to evolve adaptations to predators that would compromise its success in the open water environment. Behaviorally, *Daphnia* actively avoids macrophytes (Pennak, 1973; Dorgelo and Heykoop, 1985), which are obvious indicators of the littoral zone and the suite of predators that lurk within. By avoiding the littoral zone *Daphnia* negates the need for adaptations necessary to deter those predators. In addition to being sensitive to *Hydra* predation, *Daphnia* has been shown to be more easily captured than *Simocephalus* by damselfly naiads (Akre and Johnson, 1979; Johnson and Crowley, 1980) and the rhabdocoel flatworm *Mesostoma lingua* (Schwartz and Hebert, 1986), most probably because it is constantly swimming while *Simocephalus* is sedentary.

Members of the genus *Daphnia* have evolved a variety of morphological adaptations whose function can be explained as predator deterrence. In general, these adaptations consist of cuticular extensions to the head, tail spine, and fornices, as opposed to cuticular thickening which would increase weight and lead to major increases in basal metabolic rates. Morphological adaptations take two forms—those that have evolved as permanent structures and those that are produced in the presence of particular predators. Dodson (1984) documented the costs of the permanent adaptations of *D. middendorffiana* to predation by the copepod *Heterocope septentrionalis*. He suggested that these adaptations result in a lower reproductive rate of *D. middendorffiana* compared to that of its competitor, *D. pulex*. The cost to *D. pulex* is its elimination from those habitats containing the copepod, while *D. middendorffiana* is limited to living with its predator rather than its competitor.

The other class of morphological anti-predator adaptations of *Daphnia* are temporary features cued by chemicals produced by particular predators, a phenomenon recently termed chemomorphosis (Hebert and Grewe, 1985). Predators known to cause these effects are larvae of the phantom midge *Chaoborus* (Krueger and Dodson, 1981; Hebert and Grewe, 1985) and notonectids (Grant and Bayly, 1981; Barry and Bayly, 1985). The value of these morphological alterations in reducing predation has been demonstrated (Krueger and Dodson, 1982; Havel and Dodson, 1985), but Dodson (1984) has shown that even small cuticular extensions such as nachenzahnen reduce the intrinisic rate of increase.

Daphnia and *Simocephalus* have faced unique selective pressures for adaptations deterring predation from the predators they most frequently encounter. An important consequence of these adaptations involves the cooccurence of these cladocerans. The two species often cooccur in ponds that are large enough to support both littoral and pelagic zones (Anderson, 1974; pers. obs.). Ponds lacking either of these habitats are typically numerically dominated by one of the genera. Habitats lacking ambush predators (rock pools for example) are dominated by *Daphnia* as *Simocephalus* are excluded (Ganning, 1971; Ranta, 1979), most probably by continued competitive pressure. As observed in the aquaria study, *Simocephalus* can produce considerable populations in the absence of competitors but has smaller populations when co-occurring with *Daphnia*. These reduced populations may be due to both exploitative competition and antagonism between the two genera. Inhibitory substances have been proposed to be important in zooplankton species interactions (Frank, 1952; Seitz, 1984).

At the other habitat extreme, those ponds with a high density of macrophytic growth and the associated invertebrate predators often lack *Daphnia* but contain large populations of *Simocephalus* (pers. obs.). Species distribution is made more complex by the fact that *Daphnia* becomes a browser when planktonic food resources are limited (Mitchell and Williams, 1982). At such times *Daphnia* is particularly vulnerable to littoral and benthic predators. Therefore, the distribution of the two species is regulated by a surface area:volume ratio that takes into account not only morphometric contributions to surface area but also that due to macrophytes.

In conclusion, we have demonstrated in laboratory experiments that the outcome of competition between *Daphnia* and *Simocephalus* is regulated by the presence of *Hydra*. The broad range of morphological and behavioral adaptations to predation place *Simocephalus* at a competitive disadvantage. The divergence of life history characteristics between the species may be partially a product of competitive interactions. *Daphnia*, which faces a different suite of predators, may have evolved a unique assortment of adaptations constrained by the necessity of maintaining itself in the plankton.

Acknowledgments

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Identified Settlement Receptor Cells in a Nudibranch Veliger Respond to Specific Cue

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Abstract. Intracellular electrode recordings from sensory cells on the propodium of metamorphically competent veligers of the nudibranch *Onchidoris bilamellata* show a distinct response to a known settlement cue. Sensory cells responded to seawater conditioned with live barnacles with a slow (up to 60 s), small amplitude (3–15 mV) depolarization. Lucifer Yellow injections of these cells reveal a single, flask-shaped cell, with dendritic processes extending to the surface of the propodium. We propose that these cells are primary receptor cells that are responsible for the detection of the specific settlement cue for *Onchidoris* veligers.

Introduction

Larvae of many marine benthic invertebrates utilize a variety of environmental cues to indicate settlement and metamorphosis sites (see reviews by Crisp, 1974; Scheltema, 1974; Chia and Rice, 1978; Burke, 1983). In molluscs, one cue that often induces settlement and metamorphosis is the principal prey of the adult (Thompson, 1958, 1962; Bonar and Hadfield, 1974; Perron and Turner, 1977; Chia and Koss, 1978; Bickell *et al.*, 1981), and the nervous system is suspected in perceiving the settlement and metamorphosis cue (Baloun and Morse, 1984; Yool *et al.*, 1986). Receptor organs and cells that presumably detect these settlement/metamorphosis cues have been described in some molluscan larvae at both the light and electron microscopy levels (Bonar, 1978; Chia and Koss, 1982, 1984). However, it has never been demonstrated that these complex receptor organs actually respond to a specific cue. We are totally ignorant of the electrophysiological properties of these receptors and how they might transduce sensory information and trigger the complex behavior of settlement or the morphological changes of metamorphosis.

Todd (1979) and Chia and Koss (1988a) have shown that the nudibranch *Onchidoris bilamellata* exhibits complex settlement behavior in response to a specific cue. When metamorphically competent *Onchidoris* veligers are exposed to seawater that has been conditioned with live barnacles, they cease swimming and sink to the bottom. Upon contact with the bottom, the larval foot expands and begins rhythmical contortions. Veligers then crawl for up to 30 min and, if they do not encounter barnacles, begin swimming again. Chia and Koss (1988b) have described the morphology of receptor cells, associated with a unique ganglion, that may detect the *Onchidoris* settlement cue. The location of these cells can be readily identified under a dissecting microscope by an ovoid ciliation pattern on the sides of the propodium. *Onchidoris* thus presents a unique opportunity to characterize the electrophysiological properties of settlement receptor cells in response to a specific cue.

We have identified sensory cells in metamorphically competent *Onchidoris* veligers that respond to a known settlement cue. Intracellular electrode recordings from sensory cells on the propodium show small amplitude, slow depolarizations after the addition of seawater conditioned with live barnacles. Lucifer Yellow injections into these receptor cells reveal a single, flask-shaped cell located within a unique ganglion. This study represents the first demonstration of some of the electrophysiological properties of larval settlement receptor cells.

Materials and Methods

Egg ribbons from *Onchidoris bilamellata* (Linnaeus, 1767) were collected from Bamfield, Canada. Veliger lar-

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vae were raised according to Chia and Koss (1988a). The veligers used in these experiments were from the same cohort and random samples from the cohort were checked for metamorphic competency according to Chia and Koss (1988a). Only when the cohort reached competency did we attempt to record from a receptor cell.

Veligers were pipetted into a Sylgard (Dow Corning)-lined recording dish containing 2.5 ml of high Mg^{++}, low Ca^{++} seawater mixture (12–15°C) consisting of natural seawater, isotonic (0.33 *M*) MgCl_2, and Co^{++}-seawater in a ratio of 2:1:4.5 (v/v/v). Co^{++}-seawater consisted of 430 m*M* NaCl, 10 m*M* CoCl_2, 10 m*M* KCl, 30 m*M* MgCl_2, 20 m*M* MgSO_4, 10 m*M* TES pH 7.8 (Arkett *et al.*, 1987). We derived this bath mixture empirically and found that it abolishes most movements of the foot. Veligers were held in place by pinning the base of the velum with a cactus spine. A second spine was used to orient the veliger such that one side of the propodium and receptor field faced upward.

Conventional intracellular electrode recording techniques were used. Three *M* KCl (20–40 MO) and Lucifer Yellow CH (Sigma, tip-filled with 5% LY and back-filled with 1 *M* LiCl_2; 80–100 MO) electrodes were routinely used. Lucifer Yellow was iontophoresed into receptor cells by passing brief (1–3 s duration, up to 5 nA) hyperpolarizing current pulses for about 3–5 min. Veligers were either viewed live, or fixed in 4% paraformaldehyde in 0.2 *M* sodium phosphate buffer pH 7.4 for 1 h at room temperature. After washing briefly in the same buffer, veligers were dehydrated in a graded series of ethanol, cleared in methyl salicylate and either viewed whole or embedded in Spurr's for sectioning. Whole mounts and 2 μm sections were viewed with a Zeiss Photomicroscope II equipped with BP 450–490, FT 510 and BP 520–560 filters for Lucifer Yellow visualization.

The stimulus used to evoke settlement behavior was the same as that used by Chia and Koss (1988a) in that 25 living barnacles were soaked in filtered seawater for 24 h. One hundred μl aliquots of seawater, conditioned with the barnacle *Chthamalus dalli* (Pilsbry, 1916), were pipetted toward the veliger after the resting potential stabilized. Most veligers were tested for their response only once. Control experiments used the same aliquot volume of culture water or bath seawater.

For scanning electron microscopy, larvae were relaxed in the Co^{++}-seawater mixture described above, and processed according to the technique of McEuen (1985). Specimens were then examined with a Cambridge Stereoscan 250 SEM.

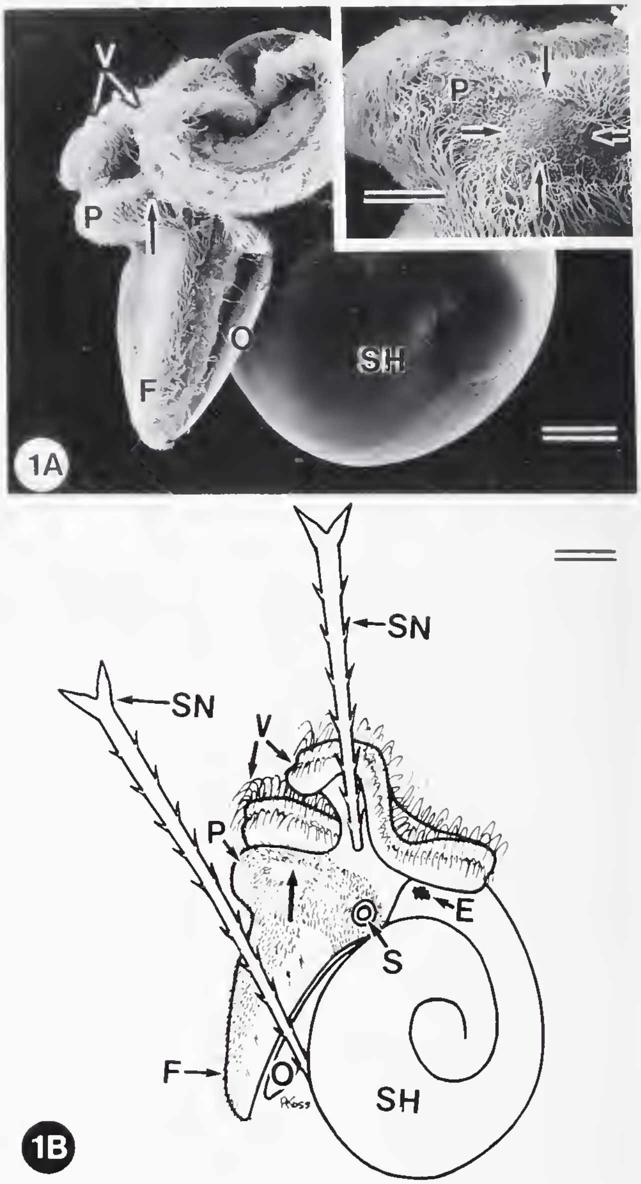

Figure 1. A. Scanning electron micrograph of competent veliger of *Onchidoris* indicating the location of one of the pair of settlement receptor fields (arrow). Bar = 50 μm. Inset shows a higher magnification of the receptor field (arrows) and propodium (P). Bar = 20 μm. B. Schematic drawing of the veliger preparation showing how veliger was pinned with cactus spines (SN). Bar = 50 μm. Velum (V), propodium (P), foot (F), operculum (O), shell (SH), statocyst (S), eye (E).

Results

While the veligers are pinned in the high Mg^{++}, low Ca^{++} seawater, pre-oral cilia beat continuously and the foot is still. The receptor fields on the sides of the propodium can be clearly seen in pinned veligers (Fig. 1A–B). Intracellular electrode recordings from the field show a mean resting potential of −57.7 mV (SE = 1.8, n = 24). No spontaneous receptor activity was observed while the preparation was in bath seawater, but an injury spike (15–20 mV amplitude, 300 ms positive phase duration) was occasionally seen upon penetration. Addition of 100

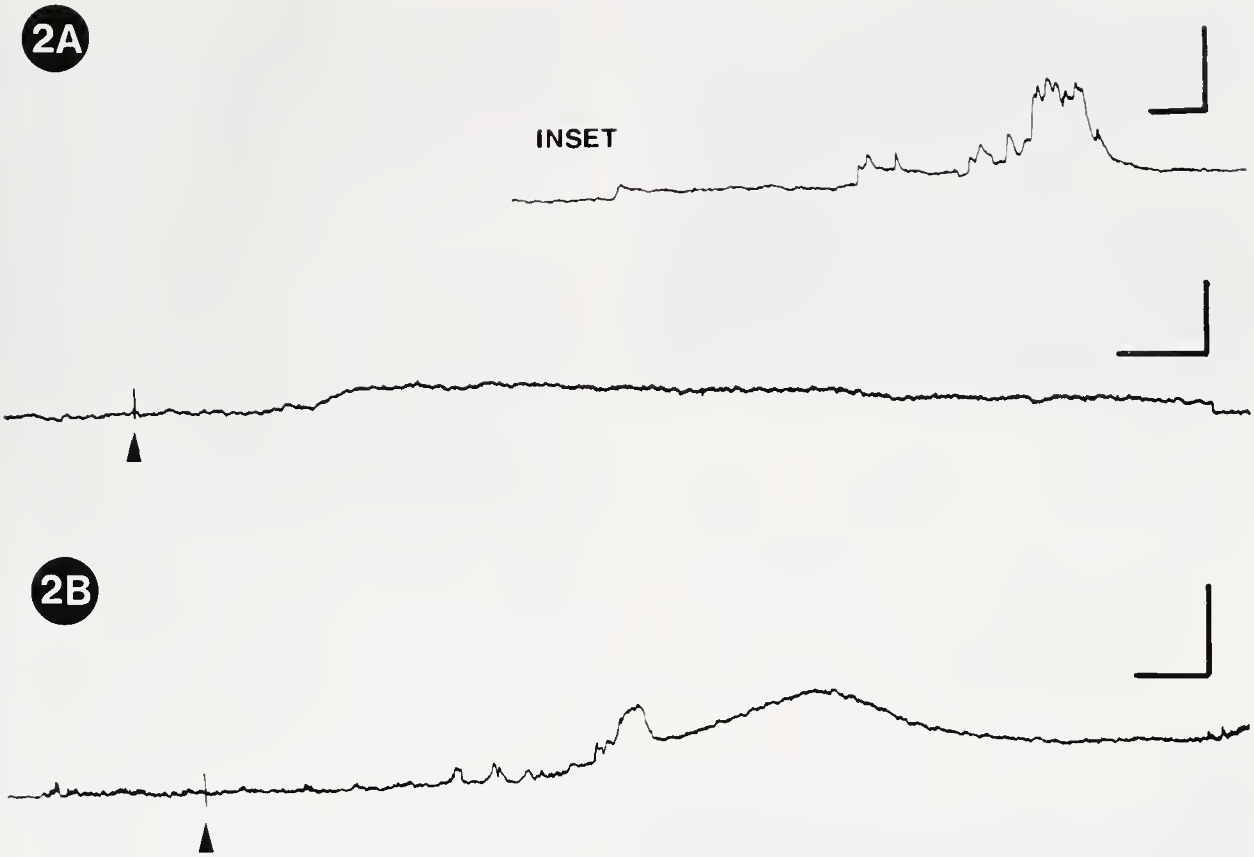

Figure 2. Intracellular electrode recordings from receptor cells in competent *Onchidoris* veligers. A. Receptor shows a response to the addition of 100 μl of barnacle water (arrow) resulting in a slow depolarization that repolarizes to resting potential. Inset shows another example where high frequency receptor potentials are superimposed on slow depolarization. B. Another example of the receptor cell response to barnacle water (arrow). Horizontal scale = 3 s (A, B), 1 s (inset). Vertical scale = 15 mV (A, B, inset).

μl of conditioned seawater results in a distinct, but somewhat variable change in the membrane potential.

The most common response to conditioned seawater is a slow depolarization, with a peak amplitude of about 5 mV (range 3–15 mV) (Fig. 2A–B). The depolarization has a rapid onset and the membrane potential repolarizes back to the resting level within 30–60 s. The initial depolarization usually occurs about 5 s after the start of the addition of the stimulus. The majority of this delay is likely due to diffusion time since the stimulus is applied several millimeters from the veliger. The depolarization is usually smooth, but occasionally small (1–3 mV) high frequency, unitary depolarizations are superimposed on these slower depolarizations (Figs. 2A–B). Injection of up to +5 nA did not elicit action potentials. Addition of a subsequent stimulus aliquot usually produced a detectable response, however, the amplitude was reduced by about 40%.

Control experiments in which we added culture seawater instead of stimulus produced no response. This experiment also served as a check for movement artifacts that could be misinterpreted as a slow change in membrane potential. Only 6 out of 24 veligers failed to show a detectable response where we were able to maintain stable resting potentials long enough to apply the stimulus. We could not detect a response either in the receptor cells or in whole animals when the veligers were bathed in 100% Co^{++}-seawater.

Lucifer Yellow injections of receptor cells reveal a single, flask-shaped cell located within the anterolateral ganglion (Fig. 3A–D). Distal dendritic processes extend toward the surface of the receptor field on the propodium (Fig. 3A–B). There was no indication of dye-coupling between receptor cells.

Discussion

We have characterized some of the electrophysiological properties of an identifiable group of cells in the anterolateral ganglia of competent veligers of the nudi-

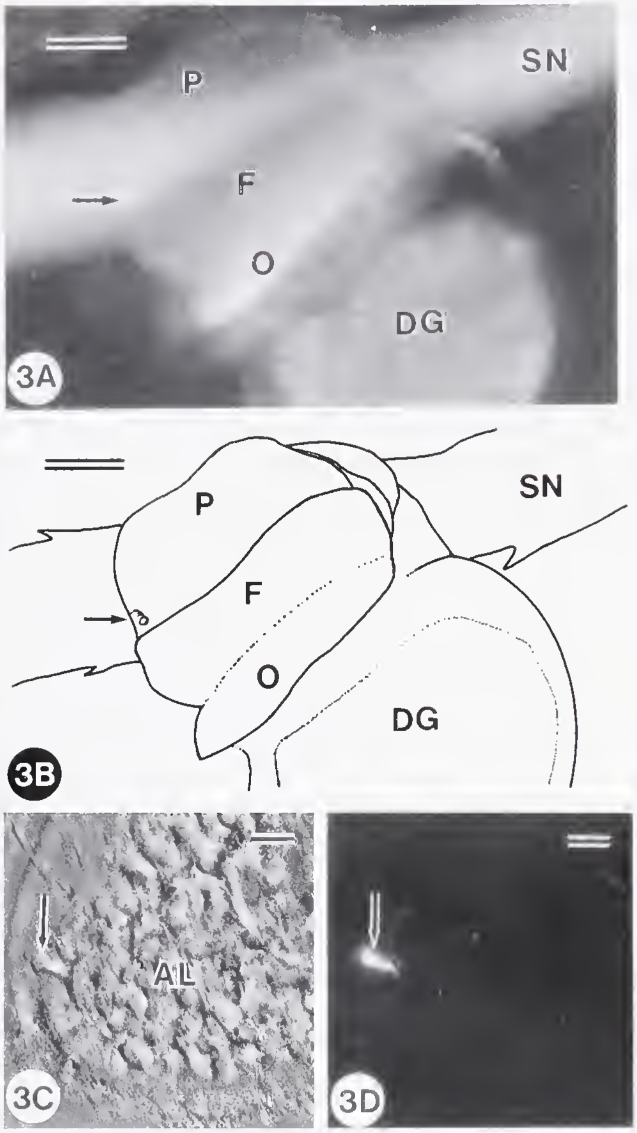

Figure 3. A. Fluorescence photomicrograph of a live veliger showing the location of Lucifer Yellow-filled receptor cell (arrow). Bar = 50 μm. Propodium (P), foot (F), operculum (O), digestive gland (DG), cactus spine (SN). B. Schematic view of A showing the orientation of the veliger and the location of the Lucifer Yellow-filled receptor cell (arrow). Bar = 50 μm. Propodium (P), foot (F), operculum (O), digestive gland (DG), cactus spine (SN). C. Oblique thick (2 μm) section through receptor field of *Onchidoris* veliger, viewed with phase contrast optics, showing a portion of a receptor cell filled with Lucifer Yellow (arrow) and the anterolateral ganglion (AL). Bar = 3 μm. D. Fluorescence photomicrograph of the same section as C showing a portion of the Lucifer Yellow-filled receptor cell (arrow). Bar = 3 μm.

branch *Onchidoris.* These experiments became possible because the size and location of these ganglia made them accessible to intracellular recording techniques. While this nudibranch veliger species possesses other probable sensory organs (Bonar, 1978; Chia and Koss, 1982, 1984), their small size and obscured location do not afford the opportunity to perform similar experiments. The anterolateral ganglion cells show a distinct response to a known settlement cue that is seawater-conditioned with live barnacles (Chia and Koss, 1988a). We propose that these cells are primary receptor neurons that detect the as yet uncharacterized settlement cue in barnacle water. We did not directly examine the response of these cells to seawater conditioned with other organisms, which does not normally induce the settlement response (Chia and Koss, 1988a). However, it remains to be determined whether the depolarizing response is absolutely specific to the settlement cue found in barnacle water.

Several pieces of evidence support our proposal that the identifiable group of cells on the larval propodium are primary receptor cells. First, Lucifer Yellow fills of these receptor cells show that they are very similar to the "sensory receptor cells" in the unique anterolateral ganglia (Chia and Koss, 1988b). The receptor cells we have described and those described by Chia and Koss (1988b) have a dendritic tree that extends to the surface of the propodium and contributes to the receptor field. Second, we have shown that even in high Mg^{++}, low Ca^{++} bathing medium, there is still a distinct receptor neuron response to the conditioned water. This medium appears to be capable of greatly reducing or blocking chemical synaptic connections, since settlement behavior (*i.e.*, ciliary arrest and foot contortions) is absent in this medium. Primary sensory cells should still respond.

We must comment on the effects of the high Mg^{++}, low Ca^{++} seawater bath on the receptor cell response. It was essential to use some anesthetic to immobilize the veligers for intracellular recordings from these small cells. The foot is active at this stage of development and settlement behavior involves strong foot contortions. However, despite the altered ionic concentration of the bath, we still see a distinct response in the receptor cells. There is some indication that the receptor potential is Ca^{++}-dependent since we could not detect a receptor cell response when the veliger was bathed in 100% Co^{++}-seawater. We do not know if the absence of external Ca^{++} exerts its effect upon a second messenger (see below) or directly through Ca^{++} channels. Regardless, it seems likely that the receptor cell response in the high Mg^{++}, low Ca^{++} seawater may be somewhat attenuated compared to the response that might occur in normal seawater.

Some of the variability in the amplitude and duration of the receptor cells' response may be due to the variable concentration of the uncharacterized ligand within the crude stimulus. The cellular response is short-lived, lasting only about 30 s, yet the stereotypic settlement behavior (*i.e.*, ciliary arrest and crawling) lasts for up to 30 min (Chia and Koss, 1988a). This suggests that a long duration complex motor pattern is triggered, even by brief exposure to the settlement cue. That subsequent additions of stimuli produced markedly reduced receptor cell responses suggests that there may be some "down regulation" of receptor sites on the cells. Further support for this idea is given by Chia and Koss (1988a) who found that veligers would not settle again within 24 hours after initial induction of settlement behavior at the onset of metamorphic competence. Another variable that we were unable to control is the level of metamorphic competency or the degree to which larvae are ready to perceive a settlement cue. There is likely some variability in competency between individual larvae within cohorts and this may explain the somewhat variable response. Furthermore, it seems likely that larvae that have delayed settlement and metamorphosis for a time after the onset of metamorphic competence may be more sensitive to settlement cues. Chia and Koss (1988a) note that larvae kept in culture greater than one week beyond onset of competence appear to settle more readily. These findings suggest that the timing of settlement and metamorphic competency and receptor sensitivity may be closely linked.

There is now some indication that settlement receptor cells in veligers may transduce sensory information in a manner similar to other olfactory neurons (Croll, 1983; Trotier and MacLeod, 1983; Getchell, 1977, 1986). In general, transduction of olfactory information involves binding of diffusable odorants to receptors on olfactory receptor cells, activation of guanine nucleotide-binding proteins (G-proteins) and adenylate cyclase, leading to the synthesis of the second messenger cyclic AMP (Pace *et al.*, 1985; Sklar *et al.*, 1986; Lancet *et al.*, 1987). Nakamura and Gold (1987) demonstrated that conductance changes are gated by both cAMP and cGMP and suggested that these changes initiate the receptor cell depolarization. That larval settlement receptor cells may function similarly is indicated by the findings of Baxter and Morse (1987). They found that larvae of the abalone *Haliotis* may be induced to settle and metamorphose when bathed in isobutylmethyxanthine (iBuMeXan), which has been shown by Kopf *et al.* (1984) to elevate intracellular cyclic AMP levels in *Haliotis* sperm. We have demonstrated here that settlement receptor cells in *Onchidoris* depolarize in response to a known settlement cue. Future experiments using the *Onchidoris* preparation may involve manipulating second messenger levels while monitoring the response of these identifiable settlement receptor cells to a specific ligand. Results from these experiments may enable us to more fully understand the cellular processes of larval settlement cue recognition.

Acknowledgments

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Ontogenic Changes in the Rates of Amino Acid Transport from Seawater by Marine Invertebrate Larvae (Echinodermata, Echiura, Mollusca)

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Abstract. Transport rates of amino acids were determined for larvae of different ages of the echiuran worm *Urechis caupo,* the gastropod *Haliotis rufescens,* the bivalve *Crassostrea gigas,* and the sea urchin *Strongylocentrotus purpuratus.* All larval forms showed an increase in the transport rate of amino acids during development. Trochophores of *U. caupo* increased their rate of net flux for each of 5 amino acids (100 n*M* each) by a factor of 1.6 and 2.2 during 1–3 days and 4–8 days, respectively, for two independent cultures. In *H. rufescens,* the maximum transport capacity (J_{max}) for alanine increased 3-fold during the 24 h required for the trochophore to develop into a veliger. In *C. gigas* veligers, there was a 9-fold increase in the maximum transport capacity for alanine during larval development from an 80 μm to a 300 μm larva. In sea urchins, the prism-stage larvae (2-day-old) had an alanine transport system with a K_t of 1.9 μM and a J_{max} of 8.1 pmol larva^{-1}h^{-1}. The kinetics of alanine transport in the pluteus-stage (4-day-old) were best described by two systems (System I: $K_t = 1.0\ \mu$M with a J_{max} of 5.6 pmol larva^{-1}h^{-1}; System II: $K_t = 132.0\ \mu M$ with a J_{max} of 8.4 pmol larva^{-1}h^{-1}). In larvae of *C. gigas,* the relationship between the rate of alanine transport and body size was described by the equation, log J_{max} (pg larva^{-1}h^{-1}) = 1.6894(X) + (−0.5937), where X is the shell length in μm. It is illustrated that the allometric increase in respiration rates, during the growth of bivalve larvae, is matched by an ontogenic increase in amino acid transport capacity.

Introduction

Most of the literature concerning the uptake of dissolved organic material (DOM) from seawater by marine invertebrates has dealt with adult forms (Jorgenson, 1976; Stewart, 1979; Ferguson, 1982; Stephens, 1988). A smaller literature describing the uptake of DOM by larvae (Bass *et al.,* 1969; Reish and Stephens, 1969; Chia, 1972; Shick, 1975; Rice *et al.,* 1980; deBurgh and Burke, 1983; Manahan, 1983a) does exist. More recently, studies have been undertaken with bacteria-free (axenic) larvae that show that the animal is the agent responsible for the observed uptake rates of DOM (Manahan *et al.,* 1983; Davis and Stephens, 1984; Jaeckle and Manahan, in press).

There is a dramatic increase in the rate of amino acid transport following fertilization of eggs, such as those of the sea urchin (Epel, 1972; Allemand *et al.,* 1984), but information is scant on how the transport rates change in larvae during later development. However, in a qualitative way it has been demonstrated that the ability for uptake of dissolved amino acids is a continuous process and occurs throughout the entire life-history, at least for bivalves. Manahan (1983b) demonstrated that the activation of transport system(s) for glycine in eggs of *Crassostrea gigas* occurs 1 h after fertilization; in the veliger and pediveliger larvae, uptake occurs across the velum; and during metamorphosis, the developing gill buds are the primary sites of uptake (Manahan and Crisp, 1983).

The present work was undertaken to determine how the rates of amino acid transport change through ontog-

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Abbreviations: DOM, dissolved organic material.

eny for a variety of marine invertebrate larval forms, representing three phyla. If the energetic contribution from DOM is to be quantitatively important throughout development, then, as larvae grow, they would have to increase their transport capacity to meet the increase in metabolic demand. Failure to do so would result in an increasingly smaller fraction of the larva's total energy needs (volume-dependent) being provided by transport processes (surface area-dependent). We report here that larvae increase their transport capacity as they increase in size during development and that, for bivalve larvae, the change in transport rates are proportional to the increase in metabolic rates, as measured by oxygen consumption.

Materials and Methods

Larval cultures

Adult sea urchins (*Strongylocentrotus purpuratus*) were purchased from Marinus Inc. (Long Beach, California) and adult echiuran worms (*Urechis caupo*) were purchased from Sea Life Supply Co. (Sand City, California). All sizes of bivalve larvae (*Crassostrea gigas*) were provided by Coast Oyster (Quilcene, Washington). Fertilized eggs of the gastropod *Haliotis rufescens* were provided by the Ab Lab (Port Hueneme, California).

Sea urchin gametes were collected from ripe adults, following injection with 0.5 *M* KCl, in 0.2 μm (pore-size, Nuclepore) filtered-seawater. Eggs and sperm were obtained from the gamete storage organs of *Urechis caupo* as previously described (Jaeckle and Manahan, 1989), except that they were not collected under aseptic conditions. Fresh 0.2 μm (pore-size) filtered-seawater was used for all cultures. All species were reared at 16–17°C, except *Crassostrea gigas* (20–21°C). During development, the water was changed either daily (mollusks), or every other day. Phytoplankton foods were not added to any of the cultures, except to the larvae of *C. gigas*.

To obtain larvae for experiments, suspensions were siphoned from the culture vessels onto mesh screens and washed with 0.2 μm (pore-size) filtered, autoclaved, seawater. The larvae were placed in a graduated cylinder and aliquots were removed for counting. A known volume of the larval suspension was then added to each experimental vial to give the required number of larvae ml^{-1}. Different larval stages, or ages, for each species were tested to determine changes in amino acid transport rates. These included: (i) the prism-stage (2-day-old) and the pluteus-stage (4-day-old) of *Strongylocentrotus purpuratus;* (ii) trochophores (1 to 8-day-old) of *Urechis caupo;* (iii) 1-day-old trochophores and 2-day-old veligers of *Haliotis rufescens;* and (iv) oyster veligers (*Crassostrea gigas*) of different sizes (80–300 μm shell length).

Kinetics of alanine transport

A known number of larvae (see figure legends) of either *Crassostrea gigas, Haliotis rufescens,* or *Strongylocentrotus purpuratus* were transferred into each of several (9–11) 20 ml vials, and sterile seawater was added until the final volume was 10 ml. One μCi of ^{14}C-alanine (168 or 170 μCi μmol^{-1}, New England Nuclear) was added to each vial. In addition, a known amount of cold carrier (^{12}C-alanine, Sigma Chemical Co.) was added to give the required substrate concentration in each vial. Depending on the species of animal (see Figs. 1, 4), these concentrations ranged from 0.5 to 500 μM. A time course experiment of 6–8 min duration (*ca.* 1 sample per min) was then carried out with each vial to measure the rate of alanine transport into larvae at each substrate concentration. At approximately 1 min intervals, a 500 μl sample of the medium (containing larvae) was removed and placed on silicone oil (Versilube F-50, General Electric Co.) in a 1.5 ml centrifuge tube. The larvae were rapidly separated (*ca.* 5 s) from the medium by centrifugation at 12,500 × *g* (Beckman Model "E" microfuge). The radioactive supernatant and oil were aspirated away, the pellet collected by cutting off the bottom of the centrifuge tube, and the larvae immediately killed with 500 μl of tissue solubilizer (Scinti-Gest, Fisher Scientific).

The larvae were then digested for at least 24 h, 5 ml of scintillation cocktail was added (Scinti-Verse, Fisher Scientific), and the radioactivity was determined with an LKB model 1211 liquid scintillation counter. Sample radioactivity was corrected, if necessary, for quenching by the addition of a ^{14}C-toluene internal standard (New England Nuclear). A least-squares regression analysis was performed on each time course experiment to determine the rate of increase in radioactivity per larva with time. The slope (radioactivity as D.P.M. larva^{-1} min^{-1}) of each time course experiment (*i.e.*, for each different substrate concentration) was corrected for the change in specific activity from the cold carrier. The rate of alanine transport, as a function of increasing substrate concentration, was calculated from these slopes and expressed as pmol larva^{-1} h^{-1}. To determine the changes in the maximum transport capacity (J_{max}) for the veliger-stages of *Crassostrea gigas*, larvae of each size were exposed to 200 μM alanine, and the rate of transport determined in a series of time course experiments as described above. Preliminary kinetic experiments showed that a substrate concentration of 200 μM caused saturation of alanine transport into *C. gigas* larvae.

Net uptake of amino acids by Urechis caupo *larvae*

Trochophore larvae of *Urechis caupo*, at different ages, were exposed to an equimolar mixture of the amino acids Ile, Leu, Met, Phe, and Val (Sigma Chemical Co.), each present at the start of the experiment at 100 n*M*. In parallel, the same amino acid mixture was added to an identical flask containing no larvae to serve as a control for any changes in substrate concentration that might have occurred due to surface adsorption. At each sampling time, a 500 μl volume of the medium was removed and passed through a 0.2 μm (pore-size) polycarbonate filter held in a 13 mm "Pop Top" filter housing (Nuclepore) to separate the seawater sample from the larvae. The seawater samples were then frozen until the amounts of individual amino acids in the medium were determined with high-performance liquid chromatography (HPLC) and fluorescence detection. Amino acids were derivatized with *o*-phthaldialdehyde (Lindroth and Mopper, 1979) and the fluorescent derivatives separated using a sodium acetate buffer system (Jones *et al.*, 1981) on an Ultrasphere ODS column (4.6 cm × 7.5 mm; 3 μm particle size). The eluent profile and HPLC equipment are described elsewhere (Manahan, 1989).

The rate of disappearance from the medium was calculated for each individual amino acid using a first-order depletion constant "k" (see Segel, 1976, p. 227). The rate of substrate depletion with time (k) was calculated from the slope of the linear regression determined for each amino acid from ln-transformed substrate concentrations. The rate of uptake for each individual amino acid was calculated by multiplying the amount of each amino acid present at time 0 by its respective depletion constant (k). Dividing this rate by the total number of larvae in the experimental flask (see legend, Fig. 3), gave the rate of uptake as fmol larva^{-1} h^{-1} for each of the five amino acids.

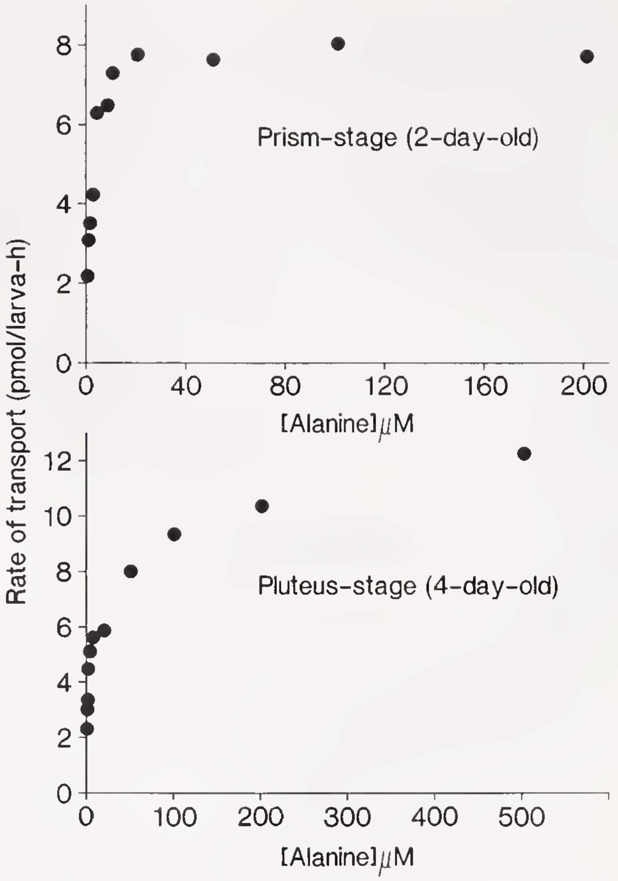

Figure 1. Ontogenic changes in the kinetics of alanine transport by larvae of the sea urchin *Strongylocentrotus purpuratus*. The upper graph shows the effect of increasing substrate concentration on the rate of alanine transport by prism-stage larvae (448 ml^{-1}). The lower graph was obtained for sibling larvae when they had developed to the pluteus-stage (408 ml^{-1}). Each data point represents a rate determined from a separate time course experiment. All values for r^2 were >0.93.

Results

The effect of increasing substrate concentration on the rate of alanine transport by *Strongylocentrotus purpuratus* larvae is shown in Figure 1. The upper graph shows that alanine transport is saturable in prism-stage larvae at substrate concentrations as low as 20 μ*M*. The experiment was repeated for sibling larvae, but two days later, when they had developed to the pluteus-stage (Fig. 1, lower graph). The rate of transport was not saturated in the pluteus-stage even when the concentration of alanine was increased to 500 μ*M*. Figure 2 shows linear transformations (Eadie-Hofstee) of the kinetic data for both prism-stage and pluteus-stage larvae. Prism-stage larvae had a transport system with a K_t of 1.9 μ*M* for alanine, and a J_{max} of 8.1 pmol larva^{-1} h^{-1} (Table I). However, the kinetics of alanine transport in pluteus-stage larvae were best described by two systems (Table I) having a combined effect of increasing the J_{max} to 12.3 pmol larva^{-1} h^{-1}. The first (System I), was a high-affinity system with a K_t of 1.0 μ*M* and a J_{max} of 5.6 pmol larva^{-1} h^{-1}. The second (System II) was a low-affinity, but higher capacity, system with a K_t of 132.0 μ*M* and a J_{max} of 8.4 pmol larva^{-1} h^{-1}. This series of experiments was repeated with a second culture of *S. purpuratus* larvae obtained from an independent spawning. A similar pattern was observed (data not shown) to that seen in Figures 1 and 2, with the appearance of a biphasic transport system in the pluteus-stage larvae.

The ontogenic changes in the net uptake rates of Ile, Leu, Met, Phe, and Val by trochophores of *Urechis caupo* are shown in Figure 3. These rates of uptake were determined with direct chemical measurements of the medium by HPLC and, hence, represent the net flux (*cf.* Johannes *et al.*, 1969). The individual flux rates for the five amino acids are plotted for larvae obtained from two independent spawnings (Fig. 3, upper and lower graph). Larvae from both cultures had an increase in the rate of net flux for each of the five amino acids. The summed

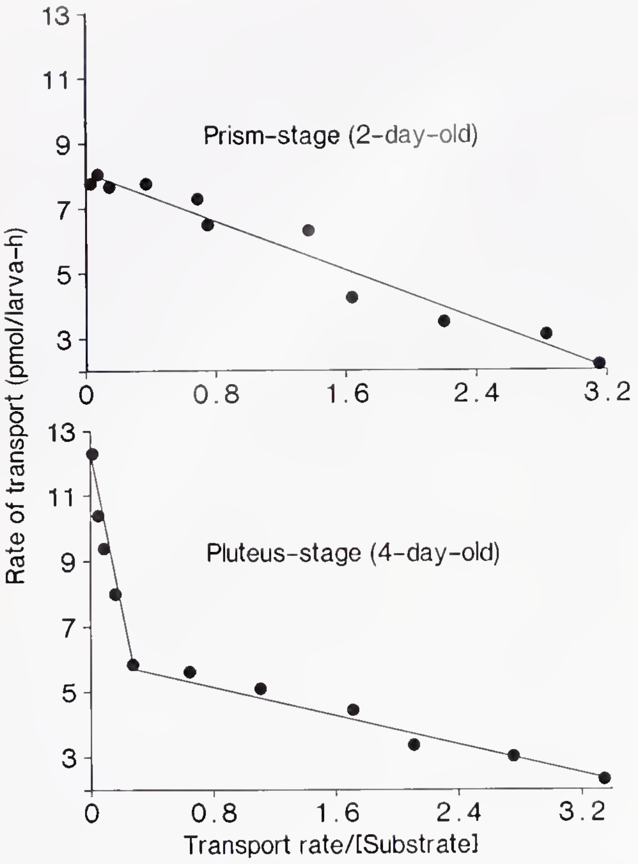

Figure 2. Eadie-Hofstee plots of the kinetic data (from Fig. 1) for alanine transport by two stages of sea urchin larvae (*Strongylocentrotus purpuratus*). The upper graph was obtained for the prism-stage larvae; the lower one for the pluteus-stage.

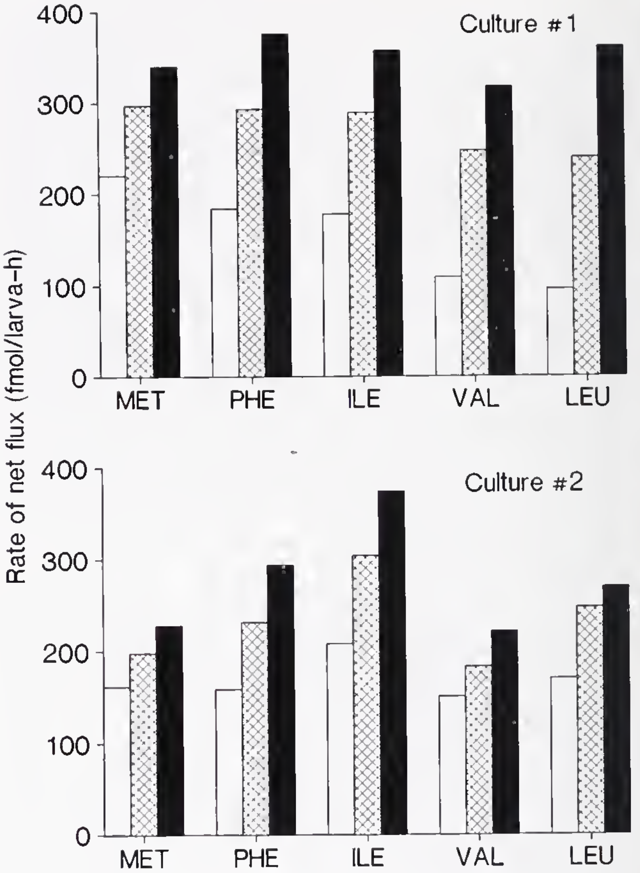

Figure 3. The change in the rate of net flux for five amino acids, as a function of age, for trochophore larvae of *Urechis caupo*. The upper and lower graphs were plotted using data from two cultures obtained from independent spawnings (nonsibling). Upper graph: the open bars, hatched bars, and solid bars represent experiments which were carried out with larvae of different ages (4-days, 6-days, and 8-days-old, respectively). Lower graph: open, hatched and solid bars represent 1-day-, 2-day-, and 3-day-old larvae, respectively. Each bar depicts a separate experiment where the depletion of substrates from the medium was determined with HPLC. Each substrate was present in a mixture at 100 n*M* each. The r^2 values for each of the ln-transformed linear regressions (n = 30) were all >0.95. The number of larvae ranged from 140 to 297 ml^{-1}, depending on the experiment.

rates of net flux were 787 fmol $larva^{-1}$ h^{-1} for 4-day-old larvae (Fig. 3, upper graph, sum of open bars) and 1751 fmol $larva^{-1}$ h^{-1} for 8-day-old larvae (sum of solid bars). Corresponding values for the second culture (Fig. 3, lower graph) were 850 fmol (1-day-old larvae) and 1386 fmol $larva^{-1}$ h^{-1} for 3-day-old larvae.

Figure 4 (upper graph) illustrates that there was a substantial increase in the transport capacity (J_{max}) for alanine during development of *Haliotis rufescens*, from the trochophore (J_{max} = 22.7 pmol $larva^{-1}$ h^{-1}) to the veliger-stage (J_{max} = 71.1 pmol $larva^{-1}$ h^{-1}). Again, these were sibling larvae for which the same kinetic experiment was repeated 1 day later, by which time the trochophores had developed into veligers. This increase in both J_{max} and K_t (see Fig. 4, lower graph) for veligers of *H. rufescens* was observed for three other cultures of larvae obtained from independently spawned batches of eggs and sperm. Larvae of *Haliotis* spp. are nonfeeding through out their entire lifespan (Crofts, 1937) and, hence, there is no major increase in biomass until after metamorphosis. There-

Table I

The appearance of biphasic kinetics for alanine transport during the development of Strongylocentrotus purpuratus *larvae*

Stage	K_t (μM)	J_{max} (pmol $larva^{-1}$ h^{-1})
Prism-stage (2-day-old)	1.9	8.1
Pluteus-stage (4-day-old)	1.0	5.6 (System I)
	132.0	8.4 (System II)

Curve fitting was calculated by microcomputer using "PC-nonlin" software.

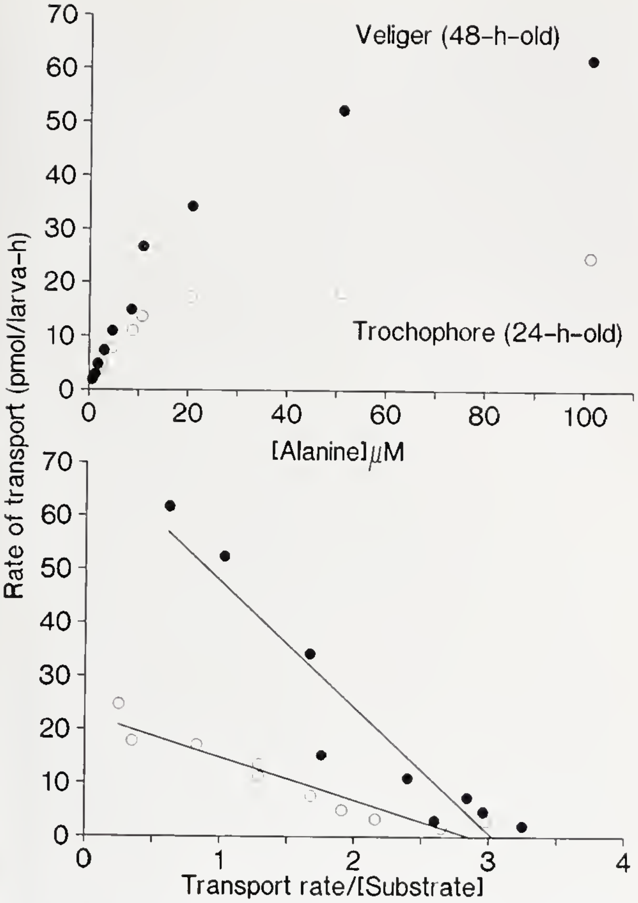

Figure 4. Upper graph (A): a comparison of the effect of increasing substrate concentration on the transport rate of alanine by two larval stages of *Haliotis rufescens*. Each point represents the rate determined from a time course experiment at that particular substrate concentration (all values for r^2 were >0.91). Lower graph (B): Eadie-Hofstee plots of the raw data from the upper graph. Trochophores (210 larvae ml^{-1}) had a K_t of 8.0 μM and a J_{max} of 22.8 pmol larva $^{-1}h^{-1}$; veligers (274 ml^{-1}) had a K_t of 22.7 μM and a J_{max} of 71.1 pmol larva$^{-1}h^{-1}$.

fore, further experiments to investigate ontogenic changes in transport rate as a function of size were carried out with feeding larvae of the bivalve *Crassostrea gigas*. Veligers of this species undergo substantial increase in size during growth when fed with cultures of phytoplankton (Helm and Millican, 1977).

Preliminary experiments showed that the rate of alanine transport by veliger larvae of *Crassostrea gigas* could be saturated at concentrations of 200 μM. This was the substrate concentration chosen for further experiments with bivalve larvae. Larvae representing seven different sizes, ranging from 80 μm to 300 μm in shell length, were chosen and their transport rates for alanine measured at 200 μM. Their respective rates of transport are shown in Figure 5 (upper graph), where each data point represents the rate obtained from a separate time course experiment (all r^2 values >0.95). The upper graph (Fig. 5) shows that there is a continuous increase in the value for J_{max} during larval development from an 80 μm larva to a 300 μm larva. The least-squares regression of the increase in the log-rate of alanine transport, as a function of increasing body size (log-shell length), is described by the allometric equation: Rate of transport (log J_{max}, pg larva^{-1} h^{-1}) = 1.6894(X) + (−0.5937), where X = shell length. The units for alanine transport in Figure 5 are presented as pg alanine larva^{-1} h^{-1} so as to permit easy

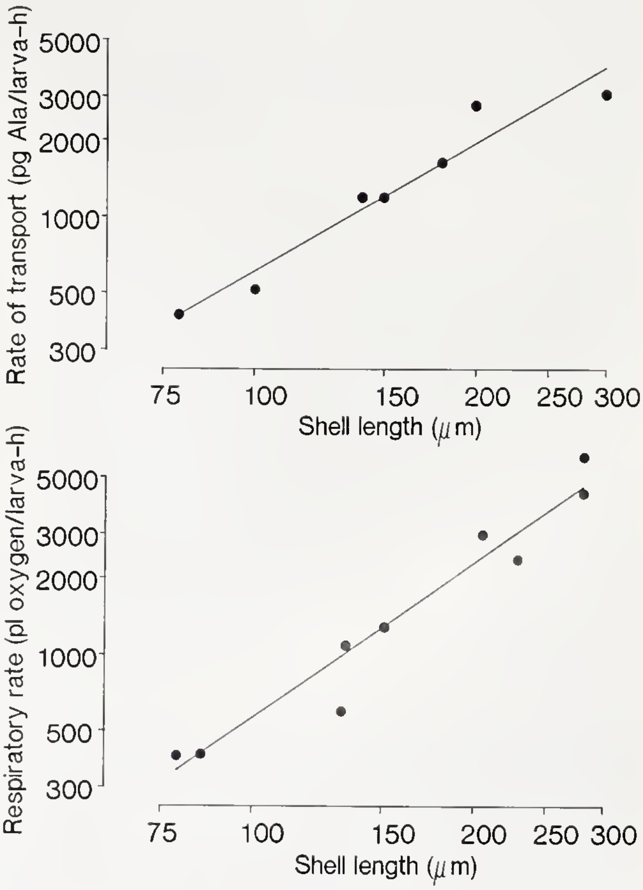

Figure 5. Upper graph (A): the logarithmic scaling of ontogenic changes in the maximum transport capacity (J_{max}) for alanine as a function of size for veliger-stage larvae of *Crassostrea gigas*. Each point represents the rate determined from a time course experiment for that particular size of larva (all values for r^2 were >0.95). The concentration of larvae used ranged from 150–400/ml, depending on size. Lower graph (B): the logarithmic scaling of respiration rate as a function of size for veliger-stage larvae of *C. gigas* (redrawn from Gerdes, 1983).

ANOVA: comparison of regressions for maximum transport capacity (J_{max}) and respiratory rate, as a function of larval size.

Source	Sum sq	d.f.	M. sq.	VR	$F_{.05}$
Combined regression	2.361	1	2.361	234.019	4.67
Between slopes	0.017	1	0.017	1.716	
Between constants	0.003	1	0.003	0.327	
Residual	0.131	13	0.010		
Total	2.512	16			

comparison to the respiration rates of similar sized larvae (1 pg protein requires 1 pl O_2 for complete combustion—Stephens, 1963). Gerdes (1983) gives data on the changes that occur in respiration rates during growth of *C. gigas* larvae. These raw data (taken from his Table III) were plotted on Figure 5 (lower graph). The least-squares regression of the increase in the rate of respiration is described by the equation: log-rate of respiration (pl O_2 larva^{-1} h^{-1} = 2.0344(X) + (−1.3163). A comparison of the slope in Figure 5A, with that of Figure 5B, illustrates the close correspondence between (i) the ontogenic increase in J_{max} for alanine transport (slope = 1.7) and (ii) the increase in respiration rates for larvae of the same species (slope = 2.0). These two slopes are not statistically different (VR = 1.7, $F_{.05[1,13]} = 4.67$, see legend to Fig. 5A, B).

Discussion

The present work was undertaken to describe how the rates of amino acid transport change during larval development. Changes were determined for trochophores of the echiuran worm (*Urechis caupo*), trochophores and veligers of the gastropod (*Haliotis rufescens*), veligers of the bivalve (*Crassostrea gigas*), and prism-stage and pluteus-stage larvae of the sea urchin (*Strongylocentrotus purpuratus*). Where J_{max} was determined, all larval forms studied showed an increase in transport capacity for amino acids as development proceeded. Obviously the surface area of larvae increases during development from the simple shape of an egg to a more complex larval form (McEdward, 1984) with extensive arms (*e.g.*, echinoderm pluteus), or large organs used for swimming (*e.g.*, molluskan velum). The simplest interpretation for the observed increase in the J_{max} for alanine transport is that, as larvae increase in surface area, the number of transport sites increases.

For bivalve larvae, the value for J_{max} increased by a factor of 9-fold as the veliger larvae of *Crassostrea gigas* increased in size from 80 μm to 300 μm. A change in the value for J_{max} can be considered as a measure of the change in the number of sites for amino acid transport. The metabolic rates for growing larvae of marine invertebrates are known to increase in direct proportion to increasing weight, with weight exponents of about 1.0 (Zeuthen, 1947; Gerdes, 1983; Sprung, 1984). Thus, unless the transport capacity for dissolved organic substrates is increased during larval growth, the relative input that the uptake of DOM would contribute to the energy needs of a larva would decrease as development proceeds. Figure 5 presents a comparison of (i) the increase in respiration rates with (ii) the increase in the transport capacity for alanine. The slopes of both the lines for alanine transport and respiratory rate are statistically similar at 1.7 and 2.0, respectively (see ANOVA in legend to Fig. 5). Thus, growing larvae scale these two processes in direct proportion. An estimate of how larvae scale (i) the absolute amount of alanine transported to, (ii) their corresponding metabolic demands, can be determined as follows. The data for the respiration rate of larvae (Fig. 5, lower graph) were taken from Gerdes' (1983) experiments, which were conducted at 25°C. From the regression line given in Figure 5 (lower graph), a 150 μm larva would have a respiration rate of 1291 pl O_2 larva^{-1} h^{-1} [log rate at 25°C = 2.0344(X) + (−1.3163), where X is shell length]. A temperature of 20°C was used during the measurements of alanine transport by larvae of *C. gigas* (Fig. 5, upper graph). Correcting the metabolic rate for temperature by assuming a Q_{10} of 2, a 150 μm larvae would have a respiration rate of 913 pl O_2 larva^{-1} h^{-1} [log rate at 20°C = 2.0344(X) + (−1.4669)]. From the regression (Fig. 5, upper graph) for the rate of alanine transport [log rate = 1.6894(X) + (−0.5937)], a 150 μm larvae would transport 1210 pg Ala larva^{-1} h^{-1}. Could this rate of alanine transport account for the metabolic rate, as measured by oxygen consumption? Usually, a 1:1 ratio for grams of amino acid (protein) and liters of oxygen is taken for these calculations (see above). However, the correct stoichiometry for the complete oxidation of alanine to ammonia requires 3 mol O_2 per mol of alanine: 1 mol Ala (89 g) requires 3 mol O_2 (at 22.4 l mol^{-1}) = 89/67.2. This gives a ratio of 1.32 g Ala = 1 l O_2 (Gnaiger, 1983). Thus, a 150 μm larva with a transport capacity of 1210 pg Ala larva^{-1} h^{-1} could balance a metabolic rate of 917 pl O_2 larva^{-1} h^{-1}, which is close to the respiratory rate for a larva of this size (913 pl O_2 larva^{-1} h^{-1}). A larva with a shell length of 150 μm was chosen for these calculation because it was in the middle of the size range studied. Similar calculations revealed that an 80 μm larva could account for 117% of its respiratory rate, with the value decreasing to 79% for a 300 μm larva. Larvae in nature are unlikely to be able to utilize 100% of their J_{max} capacity, nonetheless, the above calculations show that the J_{max} value increases during larval growth so that sufficient substrate can be supplied to the larva in order to help meet the increasing demand for energy as growth proceeds.

Our attempts to obtain accurate measurements of K_t for veliger-stage larvae proved to be difficult. Presumably, the constant experimental agitation required for reproducible sampling caused the veligers to retract their velum, the primary organ responsible for amino acid transport (Manahan and Crisp, 1983). Figure 4 shows that there is approximately a 3-fold increase in the J_{max} value for a veliger of *Haliotis rufescens*, when compared to a trochophore. However, the K_t also increased (*i.e.*, lower affinity) 3-fold, assuming a single-affinity transport system. The Eadie-Hofstee plot of the data for veligers of

H. rufescens (Fig. 4, lower graph) suggests the possibility that there may be a biphasic transport system (*cf.* the pluteus-stage larvae in Fig. 2, lower graph), but the variance in the data for the veliger-stage of *H. rufescens* did not permit detailed analysis. Only with sea urchin larvae could changes in the affinity (K_t) of the transport system be reliably determined (Figs. 1, 2). Both prism-stage and pluteus-stage larvae of *Strongylocentrotus purpuratus* have a high affinity transport system with a K_t for alanine of 1–2 μM. Yet, at the pluteus-stage a low affinity transport system appears (Fig. 2), with a K_t of 132 μM (Table I), in addition to the presence of a high affinity system ($K_t = 1\ \mu M$). The higher affinity, lower capacity, system of the pluteus-stage (Table I) had a K_t of 1.0 μM and a J_{max} of 5.6 pmol Ala larva^{-1} h^{-1}. From a substrate concentration of 500 nM, this system could transport 166 pg Ala larva^{-1} h^{-1}, equivalent (see above) to 126 pl O_2 larva^{-1} h^{-1}, or 36% of the metabolic rate (Scholander *et al.*, 1952) of developing *S. purpuratus* larvae.

Why would echinoderm larvae have a K_t for alanine of 132 μM when the natural environmental concentrations in seawater (Carlucci *et al.*, 1984; Henrichs and Williams, 1985) are thought to be approximately 100–1000 times lower? It is unlikely that, during the short time course experiments (6–8 min) used to construct the kinetic plots (Figs. 1, 2) for pluteus-stage larvae of *Strongylocentrotus purpuratus*, any substantial uptake occurred via the digestive system. In pluteus-stage larvae of another echinoid (*Dendraster excentricus*), the primary sites of uptake for a mixture of ^{3}H-amino acids were determined by autoradiography to be the epidermis and the ciliary bands (deBurgh and Burke, 1983). Very little radioactivity was localized in the esophagus and stomach. deBurgh and Burke (1983) did not see any ontogenic change in the K_t for amino acid transport in *D. excentricus*, although they emphasized that their values should be interpreted as relative, rather than absolute, because they used a mixture of amino acids and not individual substrates. For bivalve larvae, Manahan and Crisp (1983) reported similar observations with respect to the lack of uptake of ^{3}H-glycine by the digestive system of bivalve larvae (*Crassostrea gigas* and *Ostrea edulis*).

Given the natural variability in oceanic primary production rates, it is not surprising that there should be spatial and temporal variations (Mopper and Lindroth, 1982) in the amounts of DOM in different environments. However, these variations can be surprisingly large, even over relatively small scales. Smith (1986) found that the concentrations of dissolved organic nitrogen differed by a factor of 1000 in seawater samples taken just 2 m apart. On even smaller scales, multiphasic transport kinetics have been observed in isolated strains of a marine bacterium, where the K_t values for dissolved glucose range from as low as 3 nM to a high of 4 mM (Nissen *et al.*, 1984). These authors interpret their data as indicating that "microzones" of very high nutrient concentrations exist in small volumes that are relevant to bacteria, but are beyond the current technical limits of direct chemical determinations (Mopper and Dawson, 1986). A possible interpretation for the appearance of biphasic kinetics during the development of sea urchin larvae is that these larvae may occasionally experience much higher concentrations of amino acids in the microenvironments in which they live. The presence of a second, higher capacity, system for alanine transport ($K_t = 132\ \mu M$; $J_{max} = 8.4$ pmol larva^{-1} h^{-1}) would permit a higher rate of transport at any substrate concentration exceeding 260 μM, than could be achieved with the lower capacity system ($K_t = 1.0\ \mu M$; $J_{max} = 5.6$ pmol larva^{-1} h^{-1}). However, the concept that microzones containing enhanced nutrient concentrations exist in the water column is highly controversial (Jackson, 1980; Williams and Muir, 1981). Current data on the distribution of larvae in the field (Young and Chia, 1987) together with information on the composition of DOM in seawater (Wangersky, 1978), and its microspatial heterogeneity, are still too scant to permit generalizations to be made concerning ambient concentrations that might be relevant to the larvae of marine invertebrates.

At least in the case of bivalve larvae, their transport capacity for amino acids is scaled to match their increasing metabolic demands throughout development. The percent of this transport capacity that is actually utilized will depend on the ambient concentrations of amino acids in the immediate environment of the larva.

Acknowledgments

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Purification and Partial Characterization of an Autotomy-Promoting Factor from the Sea Star *Pycnopodia helianthoides*

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Abstract. Echinoderms possess collagenous connective tissues that are capable of rapid, nervously mediated changes in their tensile strength. Arm autotomy in sea stars is facilitated by a rapid decrease in the tensile strength of connective tissues in the arm base. In this study, an autotomy-promoting factor (APF) has been isolated from the fluids released by scalded or autotomizing sea stars (*Pycnopodia helianthoides*). When injected into the coleom, APF elicits a complex behavioral response that culminates within minutes in multiple arm autotomy and a generalized softening of the body wall. Injection of fluid from intact, untreated sea stars does not promote the autotomy response. APF is a water soluble, heat-labile substance derived from the body wall. It is ammonium sulphate precipitable and its activity is reduced or destroyed by several proteolytic enzymes. On the basis of its gel permeation elution pattern, APF has M_r of about 1200 Daltons. APF can be purified to a single peak of activity by reversed-phase HPLC. We conclude the substance is a peptide or has a peptide component.

Introduction

Echinoderms are unique in their possession of collagenous connective tissues that are capable of rapid, nervously mediated changes in their tensile strength (Motokawa, 1984; Wilkie, 1984). Although such mutable collagenous tissues are involved in many aspects of echinoderm biology (Emson, 1985; Wilkie and Emson, 1987; Motokawa, 1988; Wilkie, 1988), the most spectacular example of their role is in the phenomenon of autotomy, or self-mutilation. When the tensile strength of key collagenous tissues decreases rapidly and irreversibly, the result can be arm autotomy, evisceration (a complex form of autotomy in some sea cucumbers resulting in the expulsion of the viscera), or fission (a form of asexual proliferation in which the body splits into two segments).

Many sea stars will rapidly autotomize arms in response to disturbance or damage (Emson and Wilkie, 1980). Arm autotomy can be induced in the laboratory by handling, exposure to air, wounding, ligation, amputation of the tube feet, electrical shock, and chemical stimuli. Breakage usually occurs at the base of the arm, although in a few species it can occur at any level. In nature, arm autotomy probably has a defensive function by effecting release from predators such as crabs and other sea stars (Hancock, 1955, 1974). Sea stars also autotomize arms that are heavily parasitized and thus, perhaps, use autotomy as a mechanism to reduce parasite load (Riggenbach, 1903).

Chaet (1962), in the course of research on invertebrate thermal toxins, provided the first evidence that endogenous chemical factors promote autotomy in sea stars. He discovered that *Asterias forbesi* would autotomize its arms when injected with the fluid exuded by scalded conspecifics, but not when injected with fluid from nonscalded donors. He found the factor responsible for autotomy was a heat-stable, dialyzable molecule derived, apparently, from the lining of the coelomic cavity. Ob-

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servations by Davis (1967) that the parasitic gastropod, *Stylifer linckiae*, suppressed arm autotomy in the sea star, *Linckia multifora*, provide alternative, indirect evidence that arm autotomy in sea stars may be under some form of chemical control.

Chemical factors have also been implicated in the control of evisceration in sea cucumbers, a complex form of autotomy in which viscera are expelled through a rupture in the body wall. The coelomic fluid expelled by eviscerating *Sclerodactyla (Thyone) briareus* (Smith and Greenberg, 1973) and *Eupentacta quinquesemita* (Byrne, 1986) contains a factor that promotes evisceration when injected into conspecifics. Potential sources of this factor are the haemal and peritoneal systems. Smith and Greenberg (1973) suggested that the *S. briareus* evisceration factor is a small molecule on the basis of preliminary gel permeation chromatography experiments.

As Wilkie (1984) has emphasized, it is difficult to assess the physiological significance of autotomy-inducing factors in echinoderms until more is known about their chemical nature, source, site of activity, and mode of action. To begin to address some of these problems, we have undertaken a study of the chemical control of arm autotomy in the sea star, *Pycnopodia helianthoides* (Brandt 1835), a large, multi-armed species from the west coast of North America that readily autotomizes arms (Lambert, 1981).

Materials and Methods

Materials

Sea stars (*Pycnopodia helianthoides*) that ranged in size from 5 to 35 cm in overall diameter were collected by diving in Saanich Inlet near Victoria, British Columbia, Canada, and maintained at 11°C in a closed seawater system at the University of Victoria. Both male and female sea stars were used, always within a week of collection.

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri). Sephadex gels (G-25) were purchased from Pharmacia (Canada) Inc. (Baie D'Urfé, Québec). Supelcosil C_{18} columns were purchased from Supelco Canada Ltd. (Oakville, Ontario). HPLC grade trifluoracetic acid and acetonitrile were purchased from Pierce Chemical Co. (Rockford, Illinois). Artificial seawater (ASW) consisted of Jamarin U (Jamarin Laboratory, Osaka, Japan).

Preparation of crude extracts

Crude extracts of sea star autotomy-promoting factor (APF) were obtained with slight modification of Chaet's (1962) procedure. Large (25–35 cm overall diameter) sea stars were placed individually in dry 12 × 24 inch plastic autoclave bags (VWR Scientific, San Francisco, California) and immersed for 1–1.5 min in water (76°C). During immersion, the body wall of the sea star became very soft and some of the arms were autotomized. Fluid of mainly coelomic origin was released into the bag through tears that developed in the softened body wall and from wounds resulting from autotomy. This fluid was collected in chilled beakers and centrifuged at 2000 × g for 10 min. The pellet was discarded and the supernatant decanted and either bioassayed immediately for APF activity or stored (−20°C or freeze-dried). Control fluid consisted of coelomic fluid withdrawn by syringe from untreated, intact animals.

Fluid from autotomizing sea stars was also collected and tested for APF activity. Large sea stars were placed individually in autoclave bags and dropped to the laboratory floor from a height of 2 m to induce them to autotomize many of their arms. The fluid released into the bag during autotomy was collected and centrifuged and the supernatant retained for bioassay or storage.

To determine the general source of APF activity in *P. helianthoides*, the coelomic fluid was first drained (by cutting off the tips of 2–3 arms) from five sea stars (20 cm in overall diameter), and the animals quickly dissected into body wall, pyloric caeca, and stomach components (gonads were not present in these sea stars). The coelomic fluid (pooled volume approximately 500 ml) was poured into an autoclave bag, immersed in water (76°C) for 1.5 min, cooled, and bioassayed. The tissues were placed separately in autoclave bags, with a small amount of ASW, immersed in water (76°C) for 1.5 min, and the resulting extracts collected, cooled, and bioassayed.

Bioassay

Test samples and control fluids were bioassayed by intracoelomic injection into 3–6 smaller (5–10 g wet weight), intact recipient sea stars held in dishes of seawater. The amount injected was 150 μl/g wet weight of sea star (0.75 to 1.5 ml per individual). Injections were made into 3–4 different arms of the sea star. The criterion for a positive response was loss of one or more arms within 10 min of injection.

Treatment with proteolytic enzymes

Samples of crude APF were incubated for 1h at 37°C with either trypsin (100 μg/ml, pH 8.0), protease K (100 μg/ml, pH 8.0) or pronase (10 mg/ml, pH 7.2) and then bioassayed for APF activity. Controls were constructed by: (1) substituting artificial seawater for the test sample, or (2) using samples of crude APF incubated without enzyme.

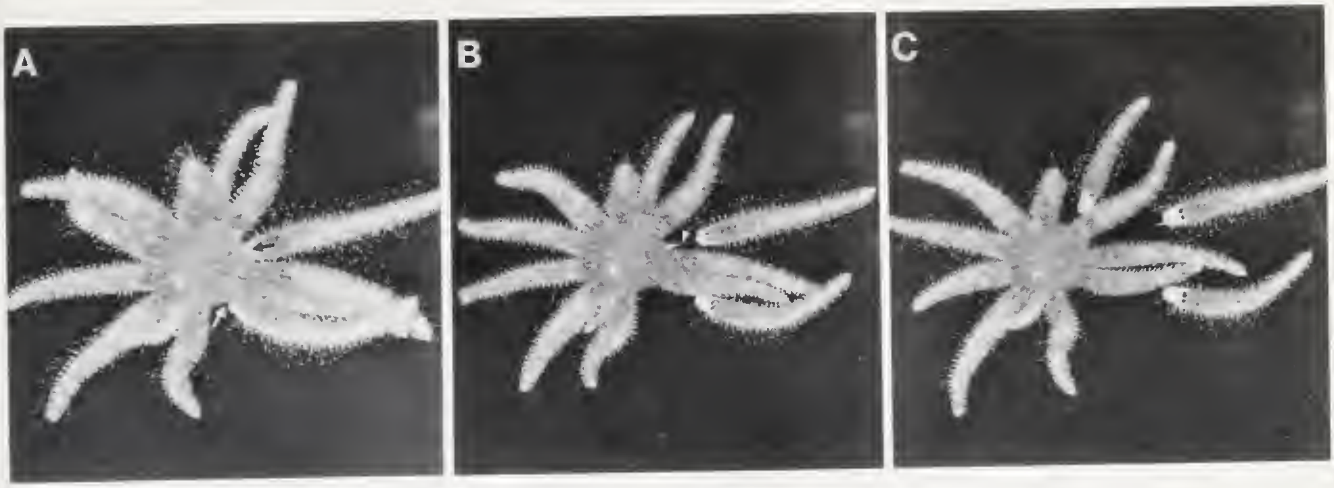

Figure 1. Response to intracoelomic injection of crude autotomy-promoting factor (APF). Onset of autotomy is apparent at the base of 2 arms (arrows) after 25 s (A); after 45 s 1 arm has autotomized completely, while the second arm remains attached to the disc by the pyloric duct only (arrowhead) (B); 3 arms have autotomized completely after 60 s and begun to crawl away from the rest of the sea star (C). ×0.5.

Purification

Gel permeation chromatography. Samples of crude APF were concentrated by freeze-drying and chromatographed on a column of Sephadex G-25 with 0.05 *M* NaCl as the mobile phase. Fractions (5 ml) were collected, pooled to 15 ml, freeze-dried, redissolved in ASW, and bioassayed. Protein concentration in the pooled fractions was estimated by the method of Lowry *et al.* (1951).

High performance liquid chromatography (HPLC). Fractions from the Sephadex G-25 column with APF activity were further analyzed by reversed-phase HPLC. This was performed on a Varian 5000 liquid chromatograph with a 1-ml sample loop, 254-nm UV absorption detector, and Supelcosil LC-18-DB column (250 mm × 4.6 mm, C_{18}, 5 μm particles). The solvent system consisted of a gradient of 0.1% trifluoracetic acid (aqueous phase) and 0.1% trifluoracetic acid in 70% acetonitrile (organic phase). Fractions (4 ml) were collected, freeze-dried, redissolved in ASW, and bioassayed.

Results

Response to crude APF

Injection of a sample of crude APF into an intact sea star (*P. helianthoides*) elicits a complex behavioral response (Fig. 1). Initially, waves of swelling travel through the body of the sea star causing transient and often localized distension of the body wall, particularly at the arm tips. A brief period of immobility often follows, during which the sea star attaches itself securely to the substratum with the tube feet. One or more arms then start to pull away from the disc as a result of centripetal movements of the tube feet. Simultaneously, the body wall tissues at the base of these arms become soft and transparent and begin to stretch apart as the arms pull away from the disc. The separation of the arm and its contents from the disc occurs in a plane passing anywhere between about the 2nd and 18th pairs of ambulacral ossicles, with larger animals autotomizing arms more distally. The tissues of the aboral body wall rupture first, then those binding the ambulacral ossicles; the pyloric duct ruptures last. By the completion of autotomy, the entire body wall of the sea star is irreversibly softened, and morbidity ensues.

Crude APF derived from heat-treated or autotomizing sea stars was equally effective in promoting arm autotomy, eliciting a full response in about 70% of the sea stars bioassayed (Fig. 2). Those sea stars that did not actually autotomize an arm invariably exhibited a strong response to injection of the crude APF that included swelling, pulling movements of the arms, and constriction of the arm bases. Mean time to initial arm autotomy in individuals injected with crude APF from heat-treated and autotomizing sea stars was 5.1 min (S.E.M. = 0.5 min, n = 10) and 19.4 min (S.E.M. = 9.4 min, n = 5), respectively. Injections of coelomic fluid withdrawn from normal, untreated sea stars never promoted autotomy (Fig. 2).

Source

APF activity was detected in body wall extracts, with 4 of the 5 sea stars bioassayed autotomizing arms within 8 min. APF activity was not present in extracts of pyloric

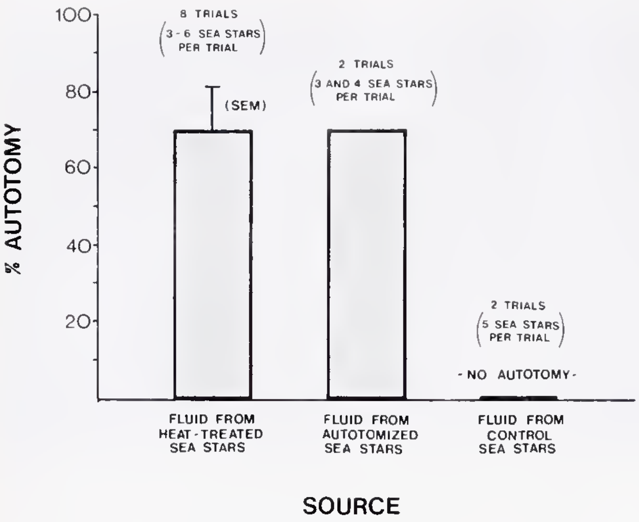

Figure 2. Results of bioassay by intracoelomic injection of fluids from heat-treated, autotomizing, and control sea stars.

caecum and stomach, and not in heated coelomic fluid (5 sea stars bioassayed for each treatment). This indicates that APF is derived from one or more of the tissues comprising the body wall.

Chemical nature of APF

pH. Crude APF from scalded sea stars was pH 6.7 (n = 4). Fluid obtained from autotomizing sea stars was pH 7.2 (n = 3). Coelomic fluid withdrawn from untreated, intact sea stars was pH 7.4 (n = 3).

Solubility. Partitioning of crude APF between aqueous and organic solvents (1 part sample in distilled water: 7 parts methanol:13 parts chloroform) resulted in APF activity in the aqueous phase but not in the organic phase or the interphase. This suggests that APF is a polar molecule and that it is not a lipid or a lipoprotein.

Stability. No activity was observed in a sample of crude APF boiled for 10 min, cooled to 4°C, and bioassayed. An aqueous sample of crude APF lost activity overnight at 4°C. Samples of freeze-dried crude APF lost activity after a few days at room temperature, but retained activity for several weeks at −20°C.

Ammonium sulphate precipitation. Precipitates of crude APF in 50% ammonium sulphate were redissolved in ASW and showed APF activity when bioassayed. Mean time to initial arm autotomy was 2.5 min (S.E.M. = 1.8 min, n = 4).

Treatment with proteolytic enzymes. Incubation of crude APF with trypsin at 100 μg/ml resulted in a smaller proportion of positive bioassays relative to the control (Table I). Incubation with protease K at 100 μg/ml also resulted in a reduced number of positive bioassays as well as a significantly longer time to initial arm autotomy (Mann-Whitney test, $P < 0.001$) in the sea stars that did autotomize arms (Table I). Incubation with pronase at 10 mg/ml completely abolished APF activity. Enzymes in ASW did not promote autotomy, while samples of crude APF incubated without enzyme retained APF activity (Table I). Thus, proteolytic enzymes reduced or abolished APF activity.

Gel permeation chromatography

Fractions with APF activity eluted just after vitamin B_{12} (MW = 1355) and before angiotensin II (MW = 1046) in a column of Sephadex G-25 (Fig. 3). The mean time to initial arm autotomy following injection with these partially purified fractions was 0.39 min (S.E.M. = 0.06 min, n = 4), considerably shorter than the response time following injection with crude APF (see above). Spectrophotometric absorbance maxima in fractions with APF activity were at 220 and 254 nm.

HPLC

Further fractionation of active fractions from the Sephadex G-25 column by reversed phase HPLC yielded a series of peaks. Only one of these peaks, eluting consistently at 45 to 48 min, had APF activity (Fig. 4).

Discussion

We have shown that an autotomy-promoting factor (APF) is present in the body fluids of both scalded and autotomizing *Pycnopodia helianthoides.* APF injected into the coelom of intact *P. helianthoides* elicits a com-

Table I

Bioassay results of treatment of crude APF with proteolytic enzymes

Treatments	% Autotomy (n = 5)	Time (min) (mean ± S.E.M.)
Crude APF (pH 7.0)[1]	100	4.8 ± 0.8
Crude APF (pH 8.0)[1]	100	7.1 ± 1.0
Crude APF + trypsin (100 μg/ml, pH 8.0)	60	8.6 ± 1.4
Crude APF + protease K (100 μg/ml, pH 8.0)	40	14.7 ± 2.8
Crude APF + pronase (10 mg/ml, pH 7.2)	0	no autotomy
ASW + trypsin (100 μg/ml, pH 8.0)[2]	0	no autotomy
ASW + protease K (100 μg/ml, pH 8.0)[2]	0	no autotomy
ASW + pronase (10 mg/ml, pH 7.2)[2]	0	no autotomy

[1] Control for presence of APF activity following incubation period.

[2] Control for effect of proteolytic enzyme alone.

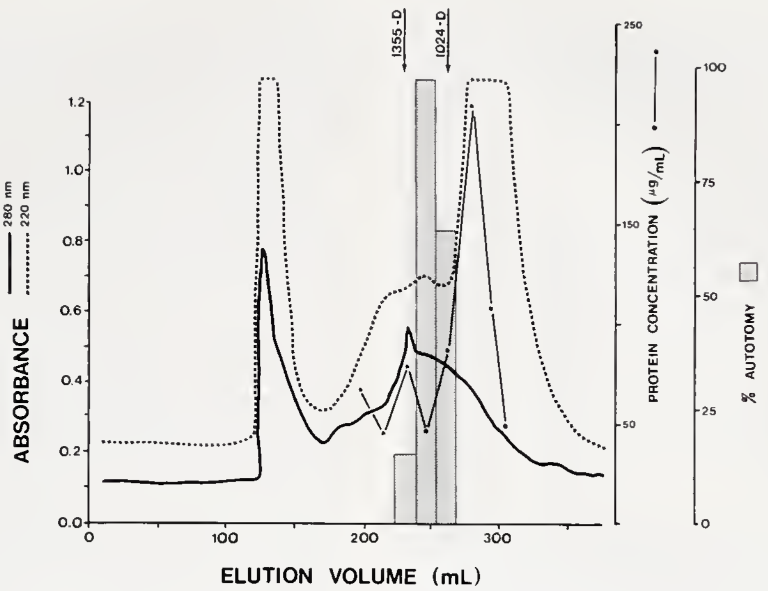

Figure 3. Elution pattern of crude autotomy-promoting factor on Sephadex G-25. Sample (100 ml) obtained from 1–2 sea stars was concentrated by freeze-drying and loaded in 3 ml on a Sephadex G-25 (fine grade) column (2.6 cm × 51 cm) equilibrated in 0.05 M NaCl at 4°C. Elution was carried out with the same solvent at a flow rate of 15 ml/h and 5 ml fractions collected. Fractions were pooled into 15 ml volumes and the protein content in each estimated by the method of Lowry. Each set of pooled fractions was then freeze-dried, redissolved in 3 ml of ASW and bioassayed (3 sea stars each). The column was calibrated with Blue Dextran 2000, vitamin B_{12} (MW = 1355), and angiotensin II (MW = 1046).

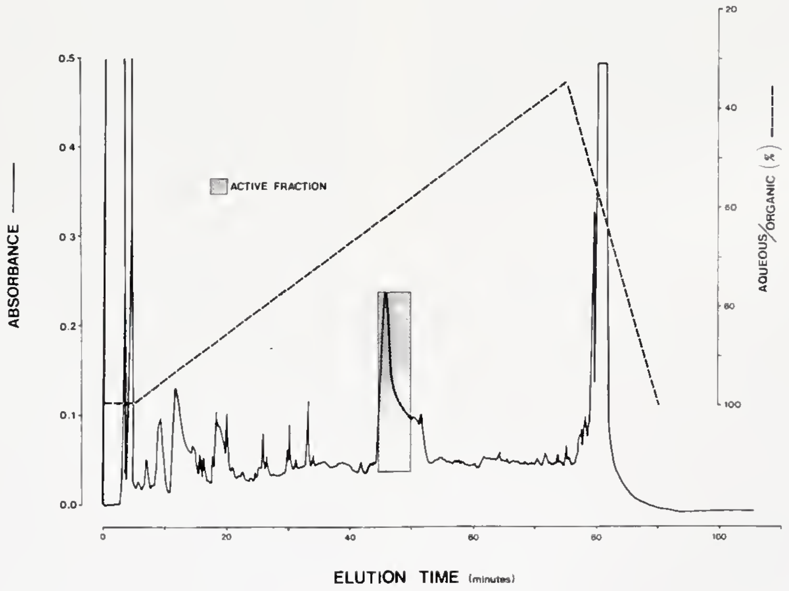

Figure 4. Typical reversed-phase HPLC elution pattern of Sephadex G-25 purified autotomy-promoting factor. An active fraction (15 ml) (elution volume 240–255 ml) from a column of Sephadex G-25 was freeze-dried, redissolved in water (1 ml) and loaded on a reversed-phase Supelcosil LC-18-DB column (250 mm × 4.6 mm, C_{18}, 5 μm particles) equilibrated in 0.1% trifluoracetic acid (aqueous solvent). After a 5-min run at 100% aqueous solvent, absorbed molecules were eluted at room temperature using a linear gradient of 100% to 35% aqueous solvent in 70 min at a flow rate of 1 ml/min. The organic solvent consisted of 0.1% trifluoracetic acid in 70% acetonitrile. Absorbance was monitored at 254 nm. Fractions (4 ml) were collected, vacuum centrifuged to dryness, redissolved in ASW (1 ml), and bioassayed (3 sea stars each). A major peak with autotomy-promoting factor activity (boxed and shaded area) was present. Mean time to initial arm autotomy was 2.5 min (S.E.M. = 0.5 min).

plex behavioral response that, after a few minutes, results in multiple arm autotomy, a generalized softening of the body wall, and morbidity. The pronounced response suggests that the amount of APF injected was an overdose.

APF from *P. helianthoides* is a polar, heat-labile substance. Fractionation by reversed-phase HPLC of Sephadex G-25 purified APF yielded a single peak of activity, indicating only a single component has APF activity. Gel permeation chromatography suggests that APF has a molecular weight of roughly 1200 Daltons. APF is ammonium sulphate precipitable, and its activity in crude extracts is reduced or destroyed by several proteolytic enzymes. It would thus appear to be a peptide or have a peptide component.

Little is known about other asteroid APFs. Chaet (1962) reported that the autotomy-promoting "toxin" from *Asterias forbesi* was a heat stable, dialyzable molecule that seemed to be derived from the parietal peritoneum or cells closely associated with it. *P. helianthoides* APF is derived from the body wall which, in our preparations, included the parietal peritoneum.

Fluids from scalded *Asterias vulgaris, A. forbesi, Solaster endeca* and *Crossaster papposus* are effective in promoting arm autotomy in both *A. vulgaris* and *A. forbesi* (Mladenov, in prep.). This suggests that APF-like molecules are present in many, if not all, sea stars and that they may have similar structures. However, unlike the *A. forbesi* "toxin," the activity of *P. helianthoides* APF is abolished after heating, indicating the probability of structural differences in the APF molecules of these particular species.

The evisceration factor from the holothurian, *Sclerodactyla briareus,* may be quite unlike *P. helianthoides* APF. Smith and Greenberg (1973) suggested that it is a very small, stable, molecule with none of the characteristics of a protein. The sources of this factor are likely to be peritoneal cells, cells within haemal vessels, or nerves associated with peritoneal or haemal tissues (Smith and Greenberg, 1973; Byrne, 1986). Chaet (1962) found that the *A. forbesi* toxin had no autotomy-promoting effect when injected into *Sclerodactyla briareus.*

The arms of *Asterias vulgaris* and *A. forbesi* are always autotomized at either side of the fifth pair of ambulacral ossicles (King, 1898; Anderson, 1956). Autotomy results from the sudden release of longitudinal muscles and connective tissues linking these ossicles, and by the softening and subsequent tearing of the tissues of the aboral body wall in the plane of breakage (Anderson, 1956). Arm autotomy in *P. helianthoides* is also facilitated by loss of tensile strength of the predominately collagenous aboral body wall at the arm base and of the collagenous structures linking the ambulacral ossicles (Wilkie, Emson, and Mladenov, in prep.). Autotomy in sea stars thus depends on the rapid loss of tensile strength of collagenous structures in the autotomy plane. Asteroid APF's must thus promote or regulate this process. The generalized softening of the body wall of *P. helianthoides* following APF injection is further evidence of this effect.

It is possible that stimulation of a sea star results in release of APF from cells in the body wall directly into the connective tissue matrix, thereby causing the softening response. Alternatively, APF may be released from the body wall into the coelom where it is transported to effector tissues. The presence of APF in the fluids of autotomized *P. helianthoides* is an indication that some APF is secreted into the coelom. Also, injection of APF into the coelom results routinely in autotomy. However, time to autotomy is longer following intracoelomic injection of APF (5.1 min, S.E.M. = 0.5 min, n = 10; this study) compared to time to autotomy following stimulation such as clamping the arm (0.4 min, S.E.M. = 0.1 min, n = 9; Mladenov, pers. obs.), which suggests that coelomic transport of APF may be incidental or secondary to direct release into the appropriate tissues.

Motokawa (1981, 1982a, b) isolated two heat-stable, low molecular weight factors from holothuroid coelomic fluid. One is methanol soluble and stiffens the connective tissue of the body wall; the other is methanol insoluble and softens the body wall. These interesting "softening" and "stiffening" coelomic factors have not been characterized, and their relationship to autotomy- and evisceration-promoting factors is unclear.

Many physiological lines of evidence implicate the nervous system in the control of the mechanical properties of echinoderm connective tissues (summarized in Motokawa, 1984, 1988; Wilkie, 1984, 1988). Morphological evidence of nervous control consists of the presence of granule-containing, neurosecretory-like cell processes in all mutable echinoderm connective tissues (Motokawa, 1984; Wilkie, 1984). Autotomy-promoting factors and coelomic "softening" and "stiffening" factors may thus prove to be neurochemicals. The physicochemical basis of variable tensility is unknown but may involve (1) changes in the charge distribution and conformation of the matrix macromolecules, (2) regulation of the extent of Ca^{++}-dependent electrostatic cross-bridging between anionic sites on matrix macromolecules, or (3) the formation of covalent cross-links between collagen fibrils and the matrix by means of an unknown Ca^{++}-dependent enzyme system (Motokawa, 1984, 1988; Wilkie, 1984, 1988; Diab and Gilly, 1984; Shadwick and Pollock, 1988). The complete characterization of APF may prove to be an important first step in resolving the physicochemical mechanism of variable tensility of echinoderm connective tissues.

Acknowledgments

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Mechanisms of Cardiovascular Compensation for Gravity in Bluefish (*Pomatomus saltatrix*)

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Abstract. Circulatory tolerance for gravity was examined in anesthetized and unanesthetized bluefish (*Pomatomus saltatrix*) tilted head up in air for short or prolonged periods. Blood pressure (BP) and heart rate (HR) were monitored using a ventral aortic catheter. During tilts in unanesthetized fish, BP decreased slightly, and tachycardia occurred. Anesthesia lowered resting BP while the response to tilting was similar to unanesthetized fish. Spinal cord transection 3 mm caudal to the obex was used to interrupt sympathetic outflow and paralyze the lower body. It produced a reduction and subsequent fall in BP during tilting. Spinal cord transection combined with vagotomy produced pronounced tachycardia, with a precipitous fall in BP with stable HR during tilting. Phentolamine mesylate (400 μg/kg) produced a fall in control BP and further reduction during tilting. When 80 μg/kg of atropine sulphate was given, tachycardia occurred and BP fell during tilting yet not below levels measured in control fish. Transient elevations of BP associated with body contractions during tilting were abolished with pancuronium bromide. Circulatory tolerance of bluefish for gravity depends upon integrity of the spinal cord and sympathetic innervation of peripheral vasculature as well as intact parasymathetic cardiac innervation. Muscular contraction of the lower body also contributes to maintaining BP during tilts.

Introduction

The cardiovascular changes that occur in humans and other mammals in response to passive head-up tilting have been the subject of many studies (see Blomquist and Stone, 1983, for review). There is typically a tachycardia with an associated fall in pulse pressure and cardiac output, and increased systemic vascular resistance. Mean blood pressure remains stable or slightly elevated. These reflex responses are thought to be mediated by afferent input from peripheral pressure and chemoreceptors and subsequent appropriate output from central autonomic cardiovascular control centers (Abboud and Thames, 1983). The overall response acts to maintain blood pressure and thereby continue perfusion to the brain, kidney, liver, and other vital organs.

In that fish live immersed in water, it may not be expected that they should have or need cardiovascular reflex responses to gravity similar to mammals. However, in earlier studies we found that bluefish (a teleost, *Pomatomus saltatrix*) were able to tolerate the physiologic stress of head-up tilting in air with only slight reduction in arterial blood pressure whereas a species of chondrichthyes (smooth dogfish, *Mustelus canis*) could not (Ogilvy and DuBois, 1982). One reason bluefish were able to avoid the orthostatic hypotension, narrowing of pulse pressure, and caudal peduncle edema observed in dogfish was thought to be the existence of a more highly organized and well-developed autonomic cardiovascular reflex system. Indeed, recent work in other laboratories has documented well-developed sympathetic and parasympathetic response systems in teleost fish which are important in maintaining vasomotor tone at rest (Smith *et al.*, 1985) and mediating cardiovascular changes during swimming (Axelsson and Nilsson, 1986; Axelsson, 1988; Priede, 1974). The purpose of the present study was to further evaluate the presence and contribution of sympathetic and parasympathetic reflexes as well as lower body muscle tone in the cardiovascular response to head-up tilting in bluefish. To do this, we used selective pharmacologic blocking agents and surgical lesions to remove one or more of the sympathetic, parasympathetic, or muscular tone components of the response.

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Materials and Methods

General handling of fish

Bluefish were caught with a hook and line in the waters around Woods Hole and kept in a tank with running salt water until used one to six days later. They were transported individually to a nearby laboratory in a small tank containing 40 l of seawater with 2 g of tricaine methanesulfonate (Aldrich Chemical Co., Milwaukee, WI). In one set of experiments no anesthetic was used. Each fish was weighed and then placed on a foam rubber pad on a V-board that was supported on a wooden trough containing a 50:50 dilution of the anesthetic solution with seawater. A small pump located in the trough pumped water through a rubber tube to a Y-tube inserted in the mouth of the fish which delivered running water to the gills. A dark piece of polyethylene was placed over the eyes of the fish to minimize visual stimuli.

Blood pressure in the ventral aorta was measured using a technique described in previous experiments (Ogilvy and DuBois, 1982). Briefly, an 18 gauge thin walled spinal fluid needle (18-T) was inserted through the floor of the mouth between the third and fourth gill arches. The obturator was removed and blood flow out of the needle confirmed adequate position in the ventral aorta. A 1-mm polyethylene tube (PE 50) was inserted into the aorta through the spinal needle. The needle was then removed with the PE 50 tubing left 4 to 5 cm into the aorta from the point of entry through the floor of the mouth. This distance placed the catheter tip 5 to 8 mm distal to the aortic valve. The PE 50 tubing was then sutured to the floor of the mouth to prevent movement during the experiment. The 1.0 meter long catheter was connected to a strain gauge manometer (Gould-Statham P23ID) to measure blood pressure. The aortic catheter was flushed intermittently with small amounts of Forster-Taggart teleost Ringer's solution containing sodium heparin (Elkins-Sinn) 20 units per ml.

The blood pressure manometer was balanced with a zero reference level at the height of the catheter tip in the ventral aorta and was calibrated with a water column. Blood pressures are expressed in mmHg. A second manometer was connected to a tube mounted on the V-board and it measured the pressure (also expressed in torr) in a column of water whose surface was at the height of the ventral aorta. Therefore, during tilting we always referred the pressures to a zero reference level at the height of the ventral aorta. Pressures were recorded continuously throughout the experiments on a direct-writing polygraph (model 5, Grass Instrument Co., Quincy, Massachusetts). Speed of response of the catheter and gauge system was measured by removing a finger tip from the end of the liquid-filled PE-50 tubing. The pressure response was 64% complete in 0.026 s, and 90% complete in 0.040 s.

Table I

Blood pressure (systolic/diastolic, mmHg) and heart rate (bpm) of resting bluefish before and after immersion in water

	In air			In water		
Fish #	Condition	P_{va} mmHg	HR bpm	Condition	P_{va} mmHg	HR bpm
21 (1975)	V-board tricaine	91/59	36	pool seawater	94/65	72
23 (1975)	V-board tricaine	59/26	30	tank seawater	53/26	30
13 (1983)	V-board no anesthesia	78/68	56	trough no anesthesia	82/68	54
Mean		76/51	41		76/53	52

Resting values in air versus water

To compare blood pressure and heart rate of bluefish in water with values obtained resting on a V-board in air, we reviewed experiments that had been done in previous years. The three in which conditions were most comparable in air and in water are as follows. The mean in air was 76/51 mmHg with a heart rate of 41 bpm and in water was 76/53 mmHg with a heart rate of 52 bpm (Table 1). In air the blood pressure gauge had its reference level set at the height of the ventral aorta, whereas when the fish was in water the gauge was rebalanced to a zero reference level at the water's surface. We found that bluefish placed under water had the same average blood pressure and heart rate as bluefish on a V-board in air provided the physiological state had not changed

Tilting protocols

Two separate tilting protocols were used in this study. In order to evaluate the blood pressure and heart rate changes to short periods of tilting, bluefish were tilted 10°, 20°, and 30° head up for 2 min, 2 min, and 5 min, respectively. This series of tilts was conducted in one group of unanesthetized fish (n = 6) as well as in a group of anesthetized fish (n = 5) in which the series of short duration tilts was repeated following the intra-arterial administration of phentolamine mesylate (400 μg/kg).

Longer duration tilts were conducted only in anesthetized fish. To establish a baseline response to 30-min tilts in anesthetized fish, heart rate and blood pressure measurements were made in 8 "control" bluefish during tilting following ventral aortic cannulation as described above. A control period of 20 min was allowed to elapse prior to the tilt. Fish were then tilted head up for 30 min

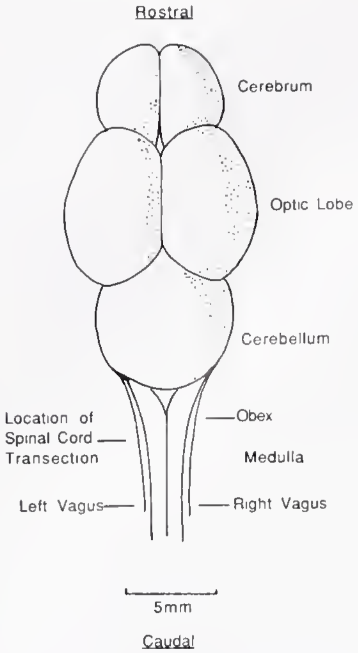

Figure 1. Dorsal view of bluefish brain, drawn from a photograph. A craniotomy exposed the cerebellum and upper cervical spinal cord. The placement of the spinal cord transection is indicated. When bilateral vagotomies were performed, the vagal fibers were transected at the same level as the spinal cord.

at an angle of 30°. A recovery period of 30 min was allowed after the tilting episode. Blood pressure and heart rate measurements were made from the polygraph record at regular intervals throughout the experiment.

To interrupt central descending sympathetic pathways and produce muscular flaccidity, we transected the spinal cord after placing the ventral aortic catheter as described above. To create spinal cord lesions, we removed the skin and musculature over the posterior one third of the skull. This exposed the central spine of the skull which was removed using an electric drill with a burr attachment (Dremel Moto-Tool, model 370-5, Racine, Wisconsin). A craniotomy was then performed using the burr attachment to carefully remove bone and expose the dura overlying the upper cervical region and lower medulla. The dura was then opened sharply and the spinal cord was exposed (Fig. 1). The vagal nerves run adjacent to the spinal cord at the level of our dissection and these fibers were carefully separated from the spinal cord using microsurgical instruments and magnification. After vagal fibers were clearly separated, the spinal cord was sharply transected using microscissors at a level 3 mm caudal to the obex (Fig. 1). Following spinal cord transection, the fish was left undisturbed for 20 min while blood pressure and heart rate were continuously monitored. The fish was then tilted head-up to 30° for 30 min. After returning to the horizontal position, blood pressure and heart rate were followed for 30 min as in fish without cord transection (control fish). In a separate set of 4 bluefish, vagal nerve fibers were transected in conjunction with the spinal cord at a level 3 mm caudal to the obex. These fish were then subjected to short tilts of 10°, 20°, or 30°.

Tilting after pharmacologic blockade

Two separate sets of experiments using pharmacologic blockade were conducted. To block alpha-adrenergic receptors, phentolamine mesylate was given intra-arterially at a dose of 400 μg/kg to each of 5 bluefish. Ten minutes were then allowed to elapse prior to a 30 min, 30° head-up tilt. As in previously described protocols, there was a 30-min recovery period after tilting. As noted above, a series of short duration tilts was also conducted in a separate set of fish after administration of phentolamine. The decision to use a dose of 400 μg/kg was based on a separate set of experiments (Fox *et al.*, 1988) where this dose of phentolamine was found to significantly alter the blood pressure response to graded doses of epinephrine.

Intra-arterial injection of atropine sulfate was used to block cardiovascular cholinergic effects in five bluefish. A dose of 80 μg/kg was used which produced a maximal heart rate (Fox *et al.*, 1988). Ten minutes after the administration of atropine, bluefish were tilted head-up for 30 min. As with phentolamine and control experiments, a follow-up period of 30 min was used.

Control experiments

In order to determine if the surgical dissection, without cord transection, had any influence upon cardiovascular function and response to tilting, we conducted a series of 2 to 5 min 10°, 20°, or 30° head-up tilts on bluefish prior to and after exposure of the spinal cord. In 4 bluefish, blood pressure and heart rate were followed with the fish on the V-board in the horizontal position for 80 min after spinal cord transection without vagotomy as described above (Table II).

Statistical analysis of data

Student's two tailed *t*-test is used to compare values of paired data for blood pressure or heart rate at each time interval during tilting to the control values recorded one min prior to tilting for the 30-min tilts. For short duration tilts, each time interval during tilting is compared to the value 10 s prior to tilting.

Results

Blood pressure and heart rate changes observed in 6 unanesthetized bluefish during head-up tilts of 10°, 20°,

Table II

Average blood pressure and heart rate before and after spinal cord transection in four anesthetized bluefish

	Time prior to transection (min)				Time after spinal cord transection (min)					
	20	10	5	1	5	10	20	40	60	80
Blood pressure (mm Hg)										
Systolic	83	76	82	84	65	68	59	54	54	56
SE	7.1	8.2	9.0	8.5	6.5	6.9	9.1	3.9	2.4	
Diastolic	53	53	54	54	47	45	43	39	37	42
SE	6.7	4.5	3.8	5.0	9.7	11.1	8.2	5.1	5.0	
Heart rate	48	53	47	44	51	42	53	42	35	48
SE	6.5	4.5	1.5	2.9	13.1	15.0	9.9	9.8	3.9	

and 30° are shown in Figure 2. There was a slight decrease in systolic and diastolic blood pressures during 10° and 20° tilts which was more pronounced during the 30° tilt. Heart rate increased during tilting, with higher angles of tilt associated with higher heart rates. The increase in rate was statistically significant during the 30° tilt (Fig. 2). A comparable set of experiments was conducted in five anesthetized bluefish (Fig. 3). Anesthetized fish as a group had a significantly lower control blood pressure compared to unanesthetized fish ($P < 0.05$ for both systolic and diastolic pressures). Resting heart rate was 58 ± 3.0 in unanesthetized and 42 ± 5.8 in anesthetized fish ($P < 0.05$). The fall in blood pressure was significant during the 30° tilt in anesthetized fish but the heart rate did not change significantly during this tilt (Fig. 3).

Thirty degree tilts for 30 min were conducted in 8 anesthetized fish (Fig. 4). The initial response was similar to that observed during 5 min, 30° tilts. After the initial 5 min of tilting, systolic and diastolic blood pressures rose and were maintained for the duration of the tilt. The blood pressure 10 min into the tilt was 76/53 mmHg which represents a 13% reduction in systolic and 10% reduction in diastolic pressure compared to the pressure 1 min prior to the tilt. A significant tachycardia developed during the first 5 min of tilting and persisted throughout the 30-min tilt.

Tilting after pharmacologic blockade

Intra-arterial administration of phentolamine mesylate in a dose of 400 μg/kg was used to block alpha-adrenergic receptors. This produced a significant reduction in resting systolic blood pressure within 5 min of administration (Fig. 5). Tilting head-up to 30° produced a significant reduction in systolic blood pressure, which recovered slightly towards the end of the tilting interval. The blood pressure 10 min into tilt was 56/43 mmHg which represents a 23% reduction in systolic and an 8% reduction in diastolic pressure compared to the blood pressure 1 min prior to tilt. Upon returning to the horizontal position after tilting, blood pressure recovered within 5 min to a mean level of 82/61 mmHg which was between the initial control blood pressure (94/55 mmHg) and the pressure 1 min prior to tilting (73/47 mmHg).

Short duration tilts of 10°, 20°, or 30° in phentolamine treated fish showed a response similar to the longer duration tilts with the greatest fall occuring in systolic blood pressure during tilting (Fig. 6). The fall in blood pressure during tilting was most pronounced for 30° tilts.

Administration through the aortic catheter of 80 μg/kg of atropine sulfate in 5 anesthetized fish produced a significant tachycardia ($P < 0.01$) within 1 min (Fig. 7) which persisted during and after tilting. This tachycardia was associated with a rise in both systolic and diastolic blood pressures when the fish were horizontal. Blood pressure fell significantly and remained low compared to pre-tilt values during the first 20 min of tilting with a slight recovery towards the end of tilting (Fig. 7). Upon returning fish to the horizontal position, blood pressure recovered within 1 min.

Tilting after spinal cord transections with and without vagotomy

Figure 8 details the response of blood pressure and heart rate prior to and after spinal cord transection with preservation of both vagal nerves (Fig. 1). Prior to transection the heart rate was 44 ± 3.9 beats/min. Five minutes after transection heart rate was 59 ± 15.5 beats/min; 20 min after transection heart rate fell to 31 ± 4.7 beats/min. Blood pressure fell from 86/59 mmHg 1 min prior to cord transection to 66/50 mmHg 20 min after transection. During the 30° head-up tilt, heart rate increased while blood pressure fell initially and continued to fall throughout the tilt (Fig. 8). During recovery after tilting,

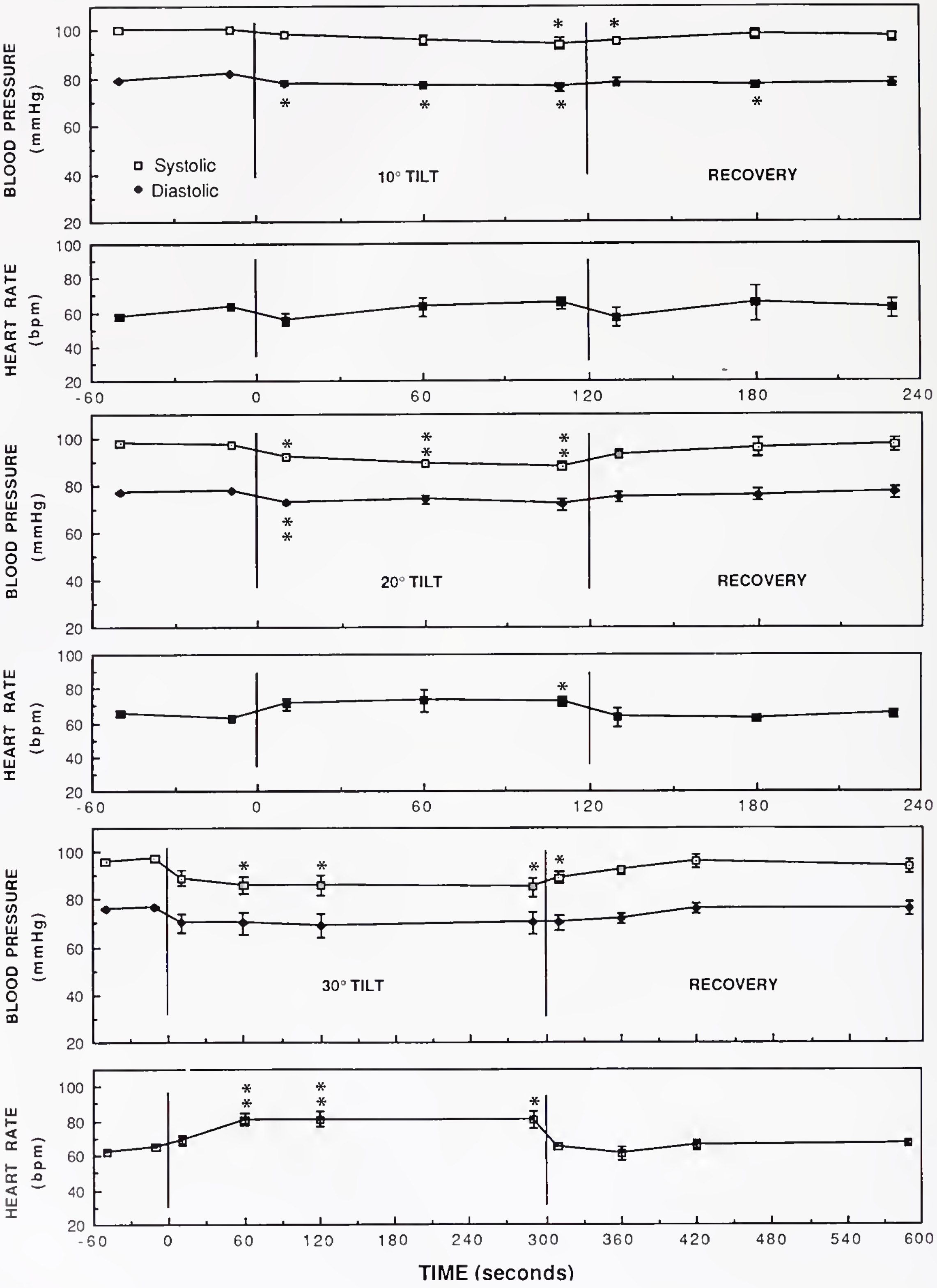

Figure 2. Response to head-up tilting in six bluefish without anesthesia. Ten and 20° tilts were maintained for 2 min, and the 30° tilt for 5 min. In this and subsequent figures the error bars represent ± one standard error of the mean using paired data to compare the values for each fish with its control value immediately before the tilt. One star indicates $P < 0.05$ and two stars $P < 0.01$.

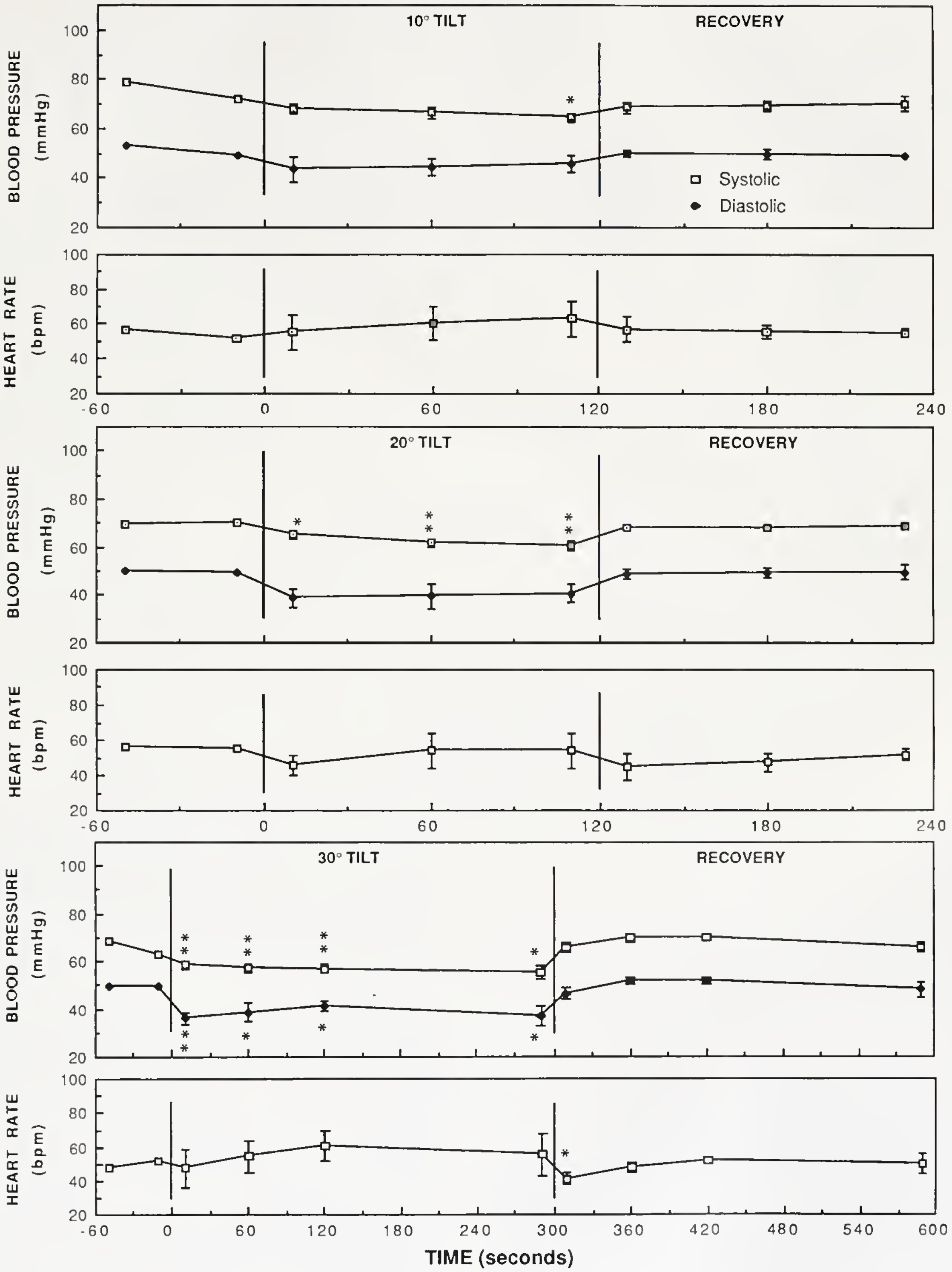

Figure 3. Heart rate and blood pressure response to 10° and 20° head-up tilts for 2 min or a 30° tilt for 5 min in 5 anesthetized bluefish.

blood pressure remained significantly lower than the pressure 1 min prior to tilt until 20 min after being returned to the horizontal position (Fig. 8).

Spinal cord transection was combined with vagotomy in four bluefish. Following this lesion, the average heart rate increased significantly and was associated with a fall

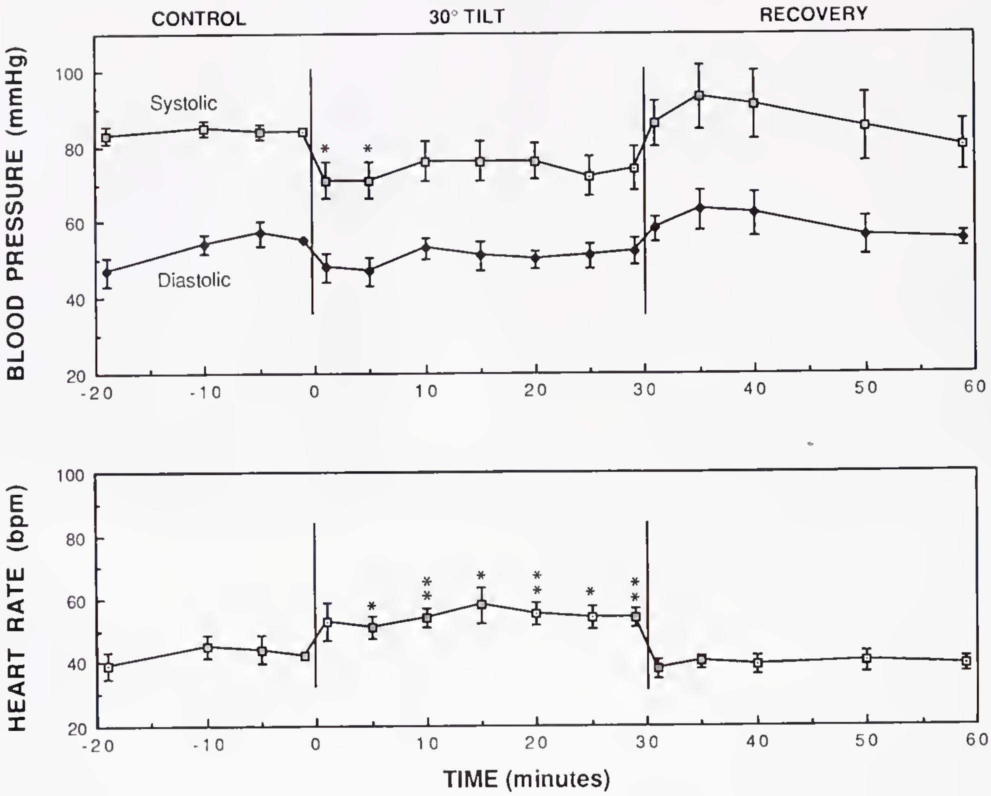

Figure 4. Blood pressure and heart rate changes before (control), during 30° tilt for 30 min, and throughout a 30-min recovery period in 8 anesthetized bluefish.

in pulse pressure as well as a slight reduction in resting mean blood pressure (Fig. 9). During tilts there was an immediate and precipitous drop in blood pressure. The decrease was greater during higher angle tilts. Pressure increased back to pre-tilt values within 10 s of returning these fish to the horizontal position. The heart rate was elevated after transection and did not increase further during tilting.

Behavioral influence on blood pressure

While bluefish rested in the horizontal position on the V-board, there were spontaneous variations in heart rate and blood pressure. When manipulations were made within the mouth there were often bradycardic episodes lasting 5–10 s. Occasional body movements consisting of alternating contractions of the body musculature were usually followed by slight (2–8 mmHg) increases in blood pressure in the ventral aorta. During head-up tilts these body movements became more frequent and often fish would exhibit 3–6 violent tail flaps that were followed by more significant increases in blood pressure. This was often true in tilts following administration of phentolamine when blood pressure was low prior to tilting. A typical example of blood pressure response to body movement is shown in Figure 10. Administration of the neuromuscular blocking agent pancuronium bromide (Elkin-Sinn, Inc.), 0.1 mg/kg intraarterially, to this fish abolished the body movements and the transient increases in blood pressure associated with body movements during tilting.

Control experiments

The response of blood pressure and heart rate to 10°, 20°, or 30° head-up tilting was the same prior to and after dissection to expose (yet not transect) the spinal cord. This analysis was conducted in 9 bluefish and the response was similar to that shown in Figure 3. Blood pressure and heart rate changes observed after spinal cord transection (no vagotomy) without subsequent tilting are shown in Table II. Blood pressure fell significantly from 84/54 mmHg 1 min prior to transection to 59/43 mmHg 20 min after transection. Between 20 and 60 min after spinal cord transection, the blood pressure remained relatively stable. The ultimate demise in 3 of the 4 fish in Table II was due to brady-arrhythmias occurring 60 to 80 min after transection. This was characterized by 2–4 s pauses in heart rate followed by resumption of the usual rate. These pauses became increasingly frequent

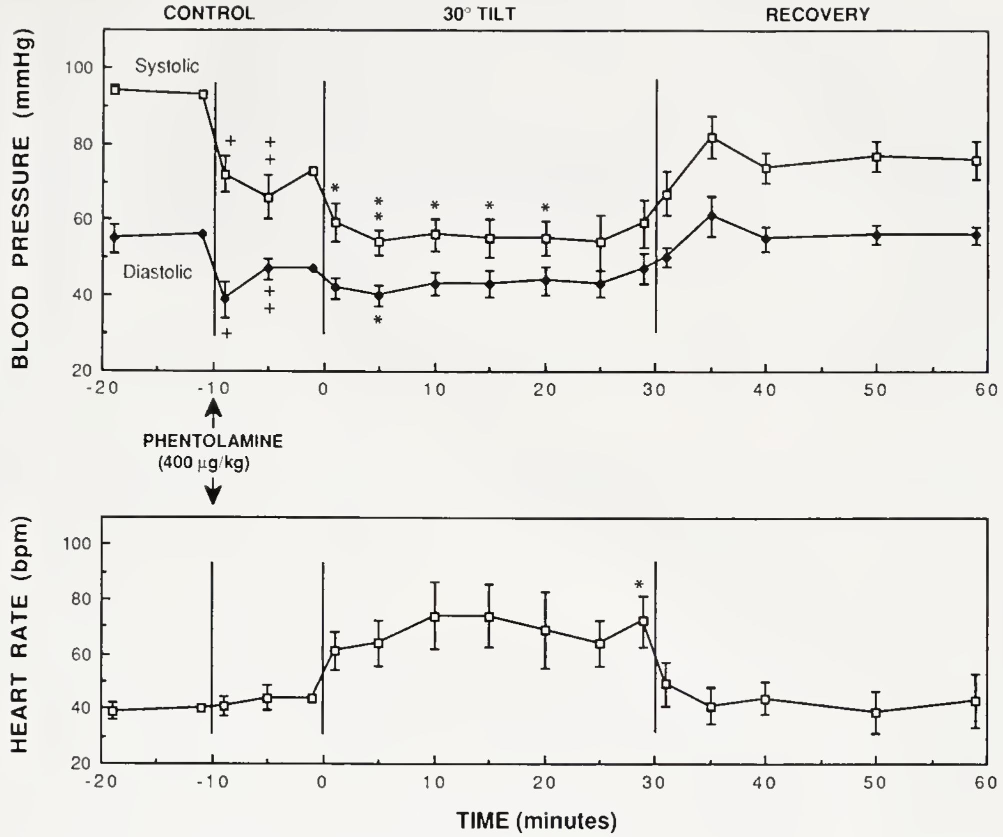

Figure 5. Phentolamine mesylate (400 µg/kg) was given in the ventral aorta 10 min prior to the 30 min, 30° head-up tilt in 5 anesthetized bluefish. In Figures 5 and 7 the single plus sign indicates $P < 0.05$ and double plus $P < 0.01$ for each fish relative to the pre-drug, or in Figure 8 pre-transection, control. The standard error bars after the drug or transection but before the tilting are paired data related to the pre-treatment control values.

over several minutes until ultimately permanent cardiac standstill occurred. These pauses were nonexistent in one bluefish in which the spinal cord was transected (vagi left intact) and 80 µg/kg of intraarterial atropine sulfate was given 10 min after transection. This fish exhibited the typical tachycardia observed in other fish following administration of this dose of atropine.

Discussion

The fact that bluefish can maintain blood pressure during head-up tilts has been documented in a previous study (Ogilvy and DuBois, 1982). However, the exact mechanism mediating this response has not previously been elucidated. In the present investigation we were able to examine the blood pressure and heart rate response during head-up tilts in unanesthetized fish, anesthetized fish, and in fish pretreated with various pharmacologic blockers or those subjected to selective or combined surgical lesions.

Unanesthetized bluefish had the highest resting blood pressure of any group studied. In addition, the resting heart rate in these fish of 55 to 65 beats/min (Fig. 2) was higher than control values of 40 to 55 beats/min observed in groups of anesthetized fish (Figs. 3, 4, 5, 7, 8). Indeed, certain general anesthetics are known to lower blood pressure and heart rate in mammals (Marshall and Wollman, 1985). The anesthetic used in our experiments does appear to have a similar effect on baseline levels of blood pressure and heart rate. This is in keeping with the findings of Houston *et al.* (1971), who documented a fall in dorsal aortic blood pressure following anesthetization of trout with 100 mg/l of tricaine methanesulphonate. Smith *et al.* (1985) found that mean ventral aortic blood pressure decreased from 5.3 kPa to 3.4 kPa while heart rate changed relatively little from 48 to 44 beats/min over 24 h after recovery from anesthesia with tricaine and surgical manipulations in codfish. It may be that despite our attempts to reduce stressful external stimuli, unanesthetized bluefish had higher degrees of sympathetic output to the heart and peripheral vasculature. However, these fish demonstrated occasional pauses of

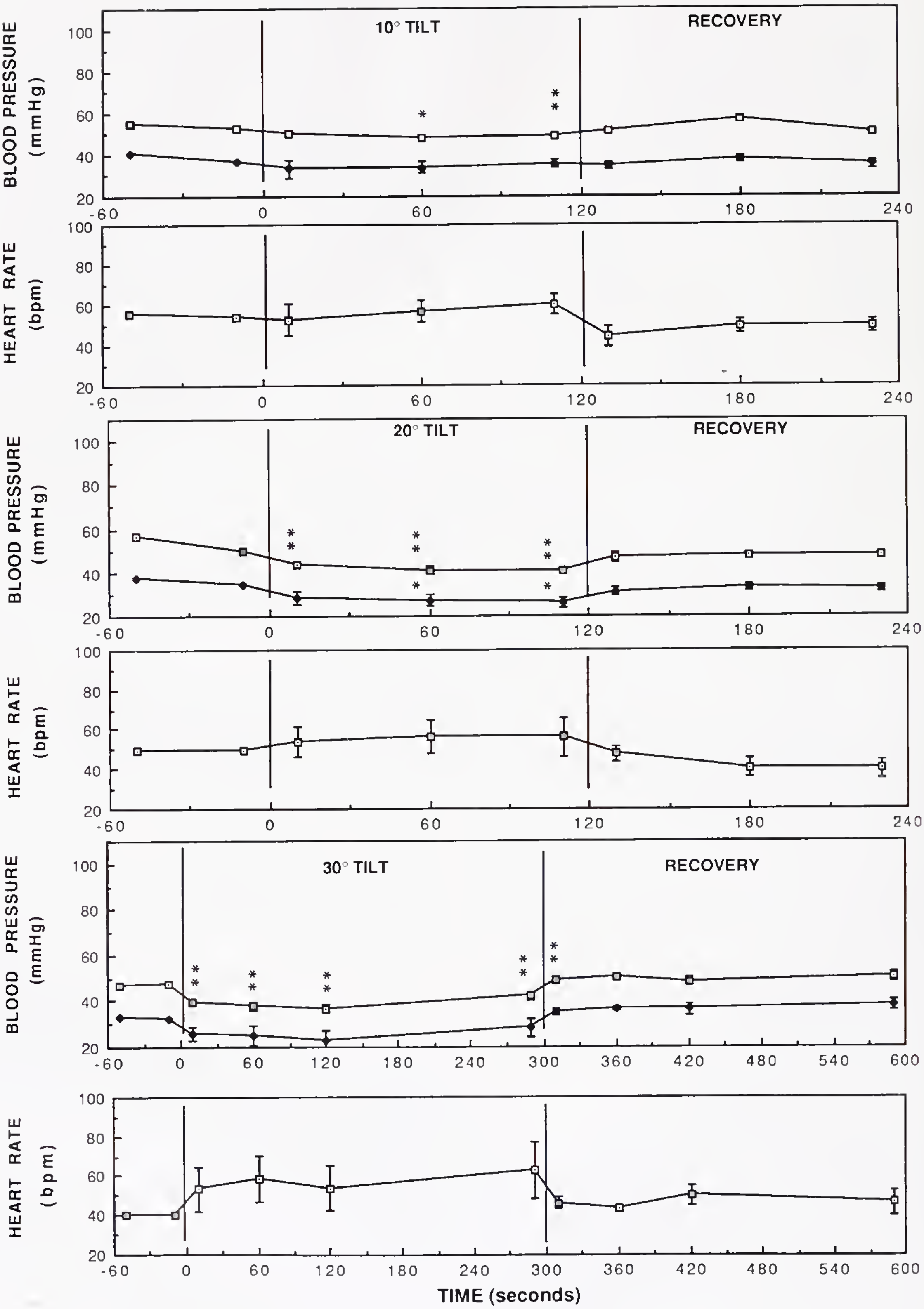

Figure 6. Head-up tilts in 5 anesthetized bluefish pretreated with 400 μg/kg of phentolamine mesylate. Ten and 20° tilts were maintained for 2 min, and the 30° tilt for 5 min.

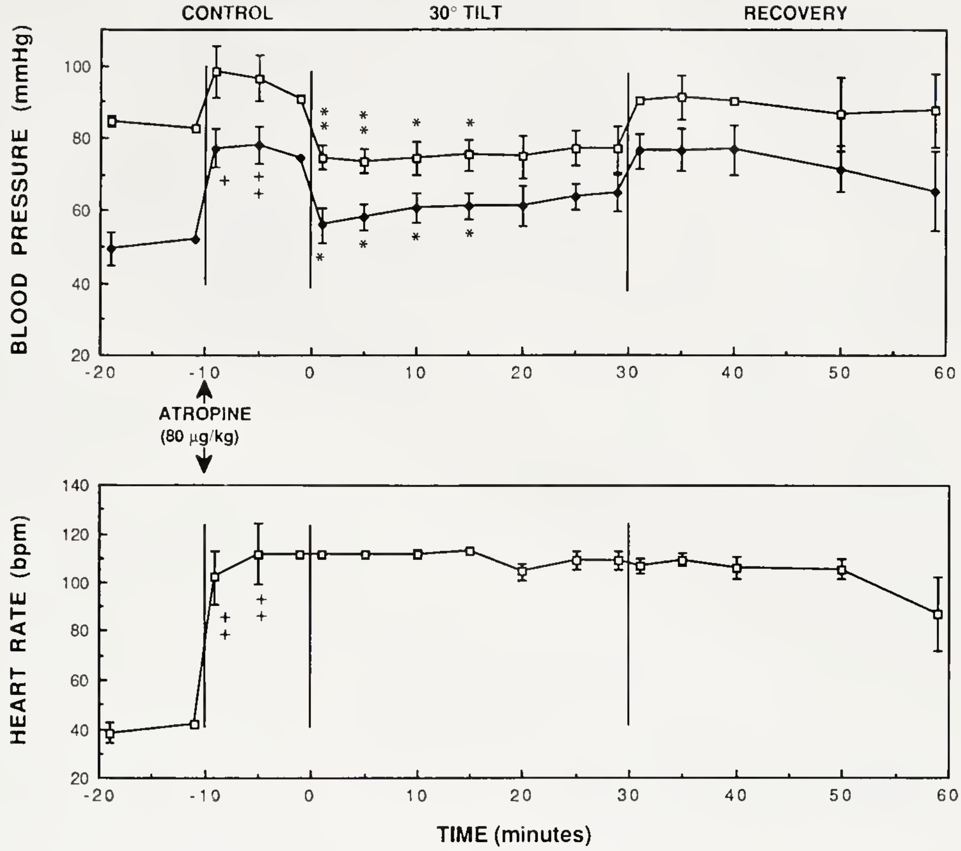

Figure 7. Atropine sulfate (80 µg/kg) was given 10 min prior to a 30° head-up tilt for 30 min in 5 anesthetized bluefish.

1–2 s in heart rate. These proved to be mediated through the vagus, implying a balance between sympathetic and parasympathetic reflex systems while the fish was at rest on the V-board. If blood pressure and heart rate were elevated due to stressful conditions, in the unanesthetized fish it may be expected that these parameters would fall over time. We observed no such reduction over time in heart rate and blood pressure.

Some general anesthetics in mammals have been implicated in resetting the baroreceptor "set point" (Marshall and Wollman, 1985). We found that although the initial blood pressure was considerably lower in anesthetized fish (Fig. 3), the response to head-up tilting was similar to unanesthetized animals (Fig. 2). In addition, anesthetized fish were able to maintain pulse pressure during the 30-min tilt (Fig. 4). We conducted the majority of tilts in anesthetized fish to allow comparison to data obtained following spinal cord transection with or without vagotomy.

Unanesthetized bluefish tolerated head-up tilts well with a tachycardia, minimal drop in blood pressure, and maintenance of pulse pressure. This response is not unlike the response described in unanesthetized humans during head-up tilts (Bie *et al.*, 1986) and lower body negative pressure (Blomquist and Stone, 1983). In mammals, this tolerance for gravity represents an integrated response due to reduced vagal outflow, peripheral sympathetic mediated arteriolar constriction and, at times, contraction of lower limb musculature to facilitate venous return to the heart (Abboud and Thames, 1983). To test the need for each of these response systems, we proceeded with experiments using sympathetic or parasympathetic pharmacologic blockade or transections of the spinal cord with and without vagotomy (Fig. 11).

The antagonist phentolamine has become a useful means of blocking alpha adrenergic mediated vasoconstriction in fish (Nilsson, 1983; Smith *et al.*, 1985; Ogilvy *et al.*, 1988). After administration of 400 µg/kg of phentolamine mesylate intra-arterially, there was a significant reduction in blood pressure (Fig. 5). During head-up tilting to 30°, there was a further significant reduction in systolic blood pressure during the first 20 min of tilting, compared to the value 1 min prior to tilt. This fall in blood pressure suggests that vasoconstriction is important in helping to maintain the blood pressure during passive head-up tilting. The tachycardia observed during

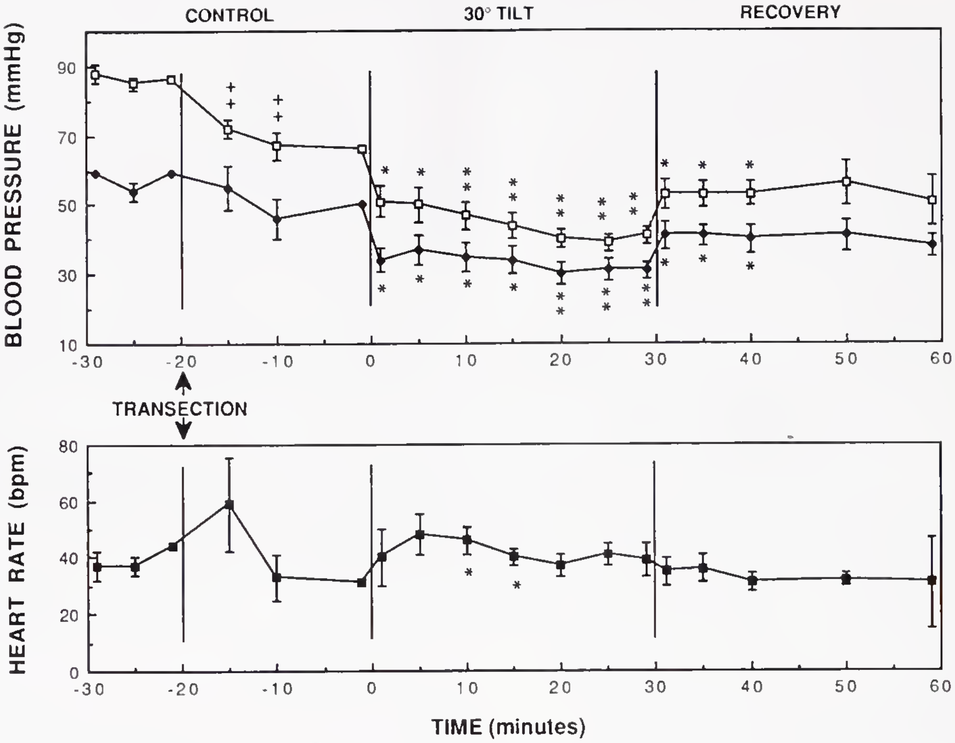

Figure 8. Transection of the spinal cord 3 mm caudal to the obex 20 min prior to a 30° head-up tilt for 30 min in 5 anesthetized bluefish.

tilts in phentolamine pretreated fish is higher than that seen in untreated fish, while pre-tilt heart rates are comparable in each of these groups of animals. The increased tachycardia in tilts with phentolamine may reflect a higher amount of baroreceptor feedback and subsequent increased suppression of vagal efferent discharge (Aboud and Thames, 1983).

The tachycardia which occurs during tilts appears to provide the fish with a useful means of attempting to maintain cardiac output (Fig. 11). This tachycardia is typical in humans in response to tilts (Bie *et al.*, 1986). We used atropine sulfate to fix the pulse at a rapid rate. As noted in results, 80 μg/kg of atropine sulfate produced a significant increase in heart rate and in blood pressure within 1 min after administration, in horizontal fish. Increased blood pressure while horizontal is presumably related to the increased cardiac output secondary to a higher heart rate. Venous return in the horizontal position is sufficient to keep up with the increased cardiac output. In the tilted position, venous return, and hence blood pressure, is diminished. Heart rate during tilts was unchanged from pre-tilt values and blood pressure fell significantly (Fig. 7). Although blood pressure was reduced, it did not approximate hypotensive levels, and as a result fish tolerated 30 min of 30° head-up tilting without any untoward effects. They swam well when returned to the holding tank. In atropine-treated fish, sympathetic arteriolar vasoconstriction as well as lower body muscle contractions can still occur, each of which contributes to an increased systemic vascular resistance and an increase in blood pressure and venous return (Fig. 11).

The spinal cord was transected 3 mm caudal to the obex to abolish both muscle tone and descending sympathetic innervation. As noted earlier, muscular contractions in our bluefish were associated with transient elevations in blood pressure (Fig. 10). This same type of behavior has been described in the black rat snake subjected to hemorrhage (Lillywhite and Smith, 1981). In these snakes wiggling movements during hemorrhage increased central venous pressure and produced a sustained increase in aortic pressure. Following spinal cord transection, bluefish became flaccid below the level of transection and were still able to make normal mouth and gill breathing motions.

Spinal cord transection with preservation of vagal fibers produced an initial tachycardia followed by a bradycardia. This type of response is typical in humans with acute complete cervical spinal cord injury where bradyarrhythmias often require atropine to prevent cardiac arrest (Lehman *et al.*, 1987). For comparison, the smooth dogfish is thought to have a less well developed cardiovascular reflex system than teleosts with relatively little resting vasomotor tone (Nilsson, 1983). These chondrichthyes tolerate total spinal cord transection with re-

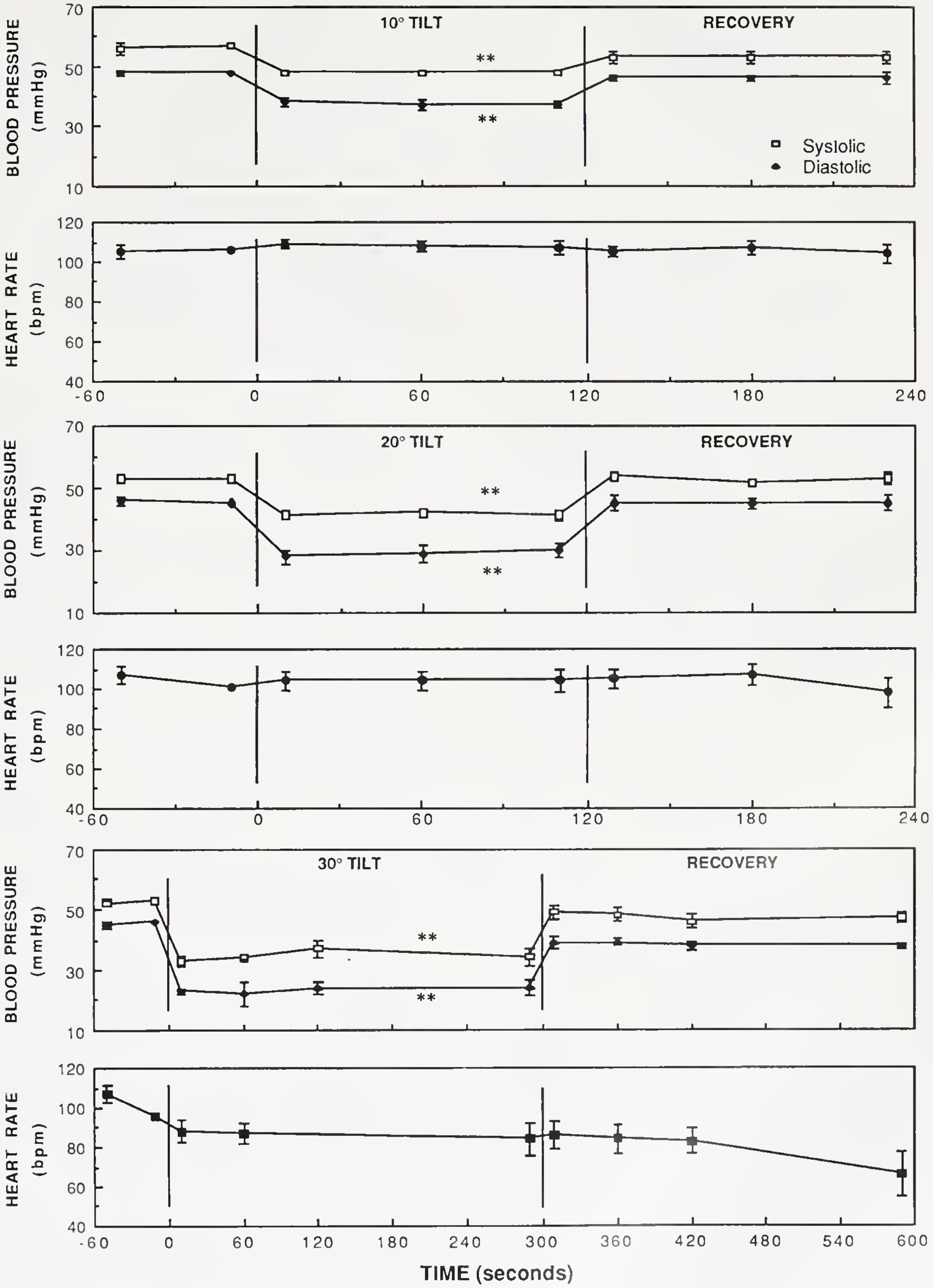

Figure 9. Blood pressure and heart rate response in 4 anesthetized bluefish, tilted 10° and 20° head-up for 2 min or 30° for 5 min, following spinal cord transection combined with bilateral vagotomy. The blood pressures during tilts are significantly lower at a level of $P < 0.001$ to $P < 0.05$ for all points during tilt compared to paired values 10 s prior to tilting.

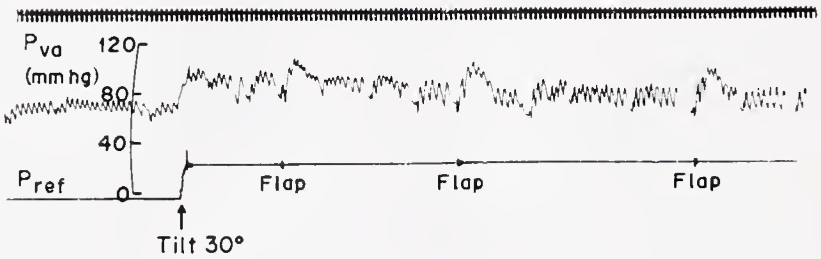

Figure 10 Typical ventral aorta blood pressure (P_{va}) response to a 30° head-up tilt in an unanesthetized bluefish pretreated with 200 μg/kg of phentolamine mesylate. Calibrations for blood pressure (in mmHg) and time marks (in seconds) are shown. The lower recording (P_{ref}) is from the reference tube set on the V-board at the level of the ventral aorta. The effective blood pressure is P_{va} minus P_{ref}. Contractions of the lower body musculature produced movements of the V-board and were recorded on the reference gauge and labelled as "flaps." Blood pressure rose in association with these movements.

moval of 1 cm of cord without any apparent change in resting heart rate or blood pressure (Opdyke *et al.*, 1972; Holcombe *et al.*, 1980). As shown in Table II, spinal cord transection without subsequent tilting produced an initial fall in blood pressure over 20 min followed by a stabilization of pressure 20 to 80 min after transection. As noted earlier, three of these four fish died of cardiac standstill, which occured with little forewarning. This was presumably based on unopposed vagal afferent input to the heart. In one fish given atropine 10 min after spinal cord transection, no pauses in heart rate were observed. In addition, in several fish where long (2 to 4 s) pauses in heart rate were observed, administration of atropine increased the heart rate and prevented subsequent episodes of severe bradycardia (Ogilvy, Fox, and DuBois, unpub. obs.).

In 5 bluefish tilted to 30° after spinal cord transection (without vagotomy), blood pressure fell despite an increase in heart rate. Theoretically, afferent impulses from the baroreceptors would still convey information to cardiovascular regulatory centers and subsequent diminished vagal output (increase in heart rate) could occur

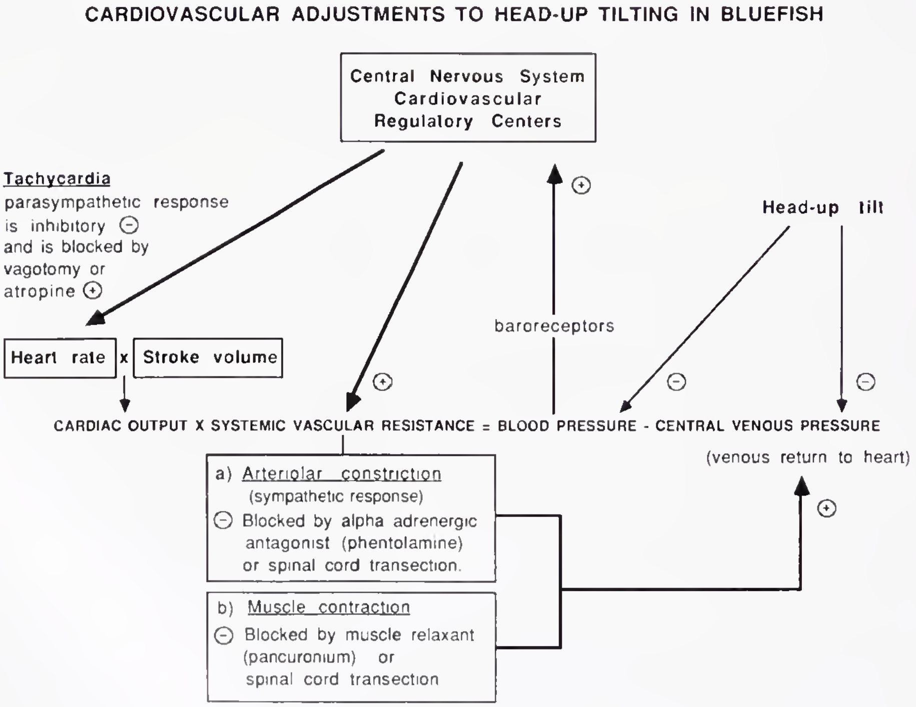
CARDIOVASCULAR ADJUSTMENTS TO HEAD-UP TILTING IN BLUEFISH
Central Nervous System
Cardiovascular
Regulatory Centers
Tachycardia
parasympathetic response
is inhibitory ⊖
and is blocked by
vagotomy or
atropine ⊕
⊕
⊕
baroreceptors
Head-up tilt
⊖
⊖
Heart rate
x
Stroke volume
CARDIAC OUTPUT X SYSTEMIC VASCULAR RESISTANCE = BLOOD PRESSURE - CENTRAL VENOUS PRESSURE
(venous return to heart)
⊕
a) Arteriolar constriction
(sympathetic response)
⊖ Blocked by alpha adrenergic
antagonist (phentolamine)
or spinal cord transection.
b) Muscle contraction
⊖ Blocked by muscle relaxant
(pancuronium) or
spinal cord transection

Figure 11. Summary of cardiovascular changes which occur in bluefish in response to head-up tilting based on experiments conducted using surgical lesions or pharmacologic blockade as indicated. See text for details.

(Fig. 11). Transection of the spinal cord disrupts sympathetic descending fibers as well as corticospinal tract fibers. Thus, constriction of peripheral arterioles or contraction of lower body musculature, two actions which could potentially increase systemic vascular resistance and contribute to maintaining blood pressure during tilts, are blocked. When muscle contractions were abolished in one fish by administration of the neuromuscular blocking agent pancuronium bromide, blood pressure fell during tilting.

Spinal cord transection was combined with vagotomy to abolish vagal control on the heart as well as muscular contraction of the lower body and descending sympathetic fibers within the spinal cord. In this group of fish, the fall in blood pressure was significant during 10°, 20°, and 30° tilts (Fig. 9). The fact that blood pressure fell and there was no change in heart rate implies total disruption of the usual reflex mechanisms which compensate blood pressure during head-up tilts.

Bluefish appear to have a well-developed set of cardiovascular reflex responses including both sympathetic and parasympathetic components. A summary of how bluefish respond to the physiological stress of head-up tilting is provided in Figure 11. During a head-up tilt, there is presumably diminished venous return (as in mammals) as well as initial alteration of blood pressure. The blood pressure change is relayed to central nervous system cardiovascular regulatory centers via afferent nerve fibers from baroreceptors located in the gill arches. Several changes occur in response to this information, the net effect of which is to maintain blood pressure and continue perfusion to vital organs. There is a tachycardia mediated by inhibition of the tonic vagal influence on the heart. This parasympathetic component of the response system can be altered by surgical vagotomy or by administration of atropine. Increased sympathetic tone causes alpha-receptor-mediated arteriolar constriction which acts to increase systemic vascular resistance. This arm of the response system can be blocked with phentolamine or by disrupting the sympathetic fibers surgically as they descend in the spinal cord. One further means of increasing systemic vascular resistance in the fish used in this study appears to be contraction of the body musculature. This should theoretically also assist venous return. This body contraction mechanism can be abolished with a neuromuscular junction blocking agent (pancuronium) or by transecting corticospinal tract fibers in the spinal cord. A similar degree of complexity of vasomotor control has been described in rainbow trout at rest (Wood and Shelton, 1980), in codfish at rest (Smith *et al.*, 1985; Axelsson, 1988) and in codfish during exercise (Axelsson and Nilsson, 1986; Axelsson, 1988). Adrenergic vascular innervation has been demonstrated in a variety of teleosts (Burnstock 1969; Nilsson, 1976, 1983). The presence of adrenergic vascular innervation implies that active vasoconstriction would be mediated through this innervation. This mechanism of response is different from that observed in dogfish where adrenergic vasoconstriction is stimulated by increases in circulating catecholamines which are liberated by angiotensin and not by neurogenic activity in the brain (Holcombe *et al.*, 1980).

It is interesting to speculate on whether the development of the tolerance of the circulatory system of teleosts for gravity developed simultaneously with that of land vertebrates, or preceded it (DuBois, 1988). Dogfish do not possess this tolerance, suggesting that the evolution of this trait occurred in Osteichthyes but not Chondrichthyes. In previous work, we have found that bluefish can accelerate with a force of 3G, and have body structures that sustain the compression caused by thrust of the tail against the inertial force of the body during rapid acceleration. The body structure also has to withstand the Pitot pressure on the front surface of the body and Bernoulli negative pressure on the "shoulder," just before the widest part of the body, while swimming through the water (DuBois *et al.*, 1976). These forces associated with swimming create circulatory transmural pressures that presumably might demand vascular responses such as those found in this study. Swimming requires an increase of cardiac output, and this increase is mediated through reflex control of the circulation.

Acknowledgments

The fish used in this study were kept in the Marine Resources Department of the Marine Biological Laboratory, Woods Hole, Massachusetts. We thank Mr. Sherman A. Goodell for catching many of the fish. Portions of this work have been presented in preliminary form (Fox *et al.*, 1987; DuBois, 1988; Ogilvy *et al.*, 1988).

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Bacterial Production of Tetrodotoxin in Four Species of Chaetognatha

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Abstract. Tetrodotoxin (TTX) producing bacteria have been isolated from four species of planktonic chaetognaths: *Flaccisagitta lyra, Parasagitta elegans, Zonosagitta nagae,* and *Eukrohnia hamata.* TTX production in bacterial isolates from the four species of venomous chaetognath species was indicated by neuroblastoma cell culture bioassay and high performance liquid chromatography. A common species of marine bacterium, *Vibrio alginolyticus,* was identified as most likely responsible for the production of TTX. Extracellular TTX in the culture medium at concentrations of 280–790 pg/ml indicated that *V. alginolyticus* may be sufficiently capable of replenishing venom TTX within 24 h. It is suggested that this bacterium lives associated with these chaetognaths and is responsible for the production of the TTX in chaetognath venom.

Introduction

Observations of arrowworms paralyzing copepods in the laboratory (Parry, 1944; Nagasawa, 1985) and investigations of their raptorial morphology (Bieri *et al.*, 1983; Thuesen and Bieri, 1987) indicated that these primary carnivores use venom to enhance the likelihood of successful prey capture. Suppositions of their venomous nature were confirmed with the recent discovery that the sodium channel blocking agent tetrodotoxin (TTX) is present in the heads of a diverse number of chaetognaths (Thuesen *et al.*, 1988). Since many species of marine bacteria are evidently capable of producing TTX (Simidu *et al.*, 1987; Tamplin *et al.*, 1987; Yotsu *et al.* 1987), the present study was undertaken to determine if bacteria may be responsible for the production of TTX in chaetognath venom.

Materials and Methods

Zooplankton sampling was carried out on the *R/V Hakuho Maru,* Ocean Research Institute, University of Tokyo, cruise KH-87-4 in the Kuroshio and Oyashio mixing area off the northwestern coast of Japan. A 160-cm ORI net hauled obliquely to 1000 m and closing 56-cm MTD nets hauled horizontally at determined depth intervals to 700 m were used to collect samples. Animals were removed from the plankton sample immediately after collection, identified, and transferred to sterilized seawater. The chaetognaths *Flaccisagitta lyra* (Krohn), *Parasagitta elegans* (Verrill), *Zonosagitta nagae* (Alvariño), and *Eukrohnia hamata* (Möbius) were used as sources for the isolation of bacteria.

Immediately following removal from the plankton sample, chaetognaths were rinsed once more in sterilized seawater, decapitated, and the heads transferred to microhomogenizers. Heads were lightly homogenized either singly or in groups of five or ten. Some homogenates were prepared with the addition of one or two drops of sterilized seawater. Agar plates of $\frac{1}{5}$ strength ZoBell's 2216 medium (90% aged seawater) were then inoculated with the homogenate and incubated at 20°C. Since it is known that bacteria of the genus *Vibrio* are producers of TTX (Simidu *et al.*, 1987) and commonly associated with zooplankton (Simidu *et al.*, 1971), a survey of a variety of the bacterial colonies which had formed on the plates was carried out using the Hugh-Leifson test to select facultative anaerobes for analysis to determine TTX production.

Since all of the isolated *Vibrio* strains were found to have the property of "swarming on solid media," many of the possible *Vibrio* species were tentatively eliminated and the identification process simplified (Baumann and Schubert, 1984; West and Colwell, 1984). To identify the bacteria responsible for TTX production, we performed

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the following tests: growth at 40°C, tolerance to 10% NaCl, utilization of sucrose, amylase production, gelatinase production, arginine dihydrolase, and Voges-Proskauer test of acetoin production. *Vibrio alginolyticus* (NCMB 1903) was used as a standard for comparison throughout the identification process.

Media for batch culture of bacteria was prepared of the following components: NaCl, 26.38 g; $MgSO_4 \cdot 7H_2O$, 2.46 g; K_2HPO_4, 1.0 g; Bacto-peptone (Difco), 5.0 g; yeast extract, 1.0 g; and distilled water, 1.0 l, adjusted to pH 7.5. Bacterial strains were inoculated into 250 ml of culture medium and incubated at 30°C with shaking until cells attained stationary growth phase ($\approx$24 h). Cells were harvested by centrifugation at 7000 rpm and washed once with 0.3 *M* NaCl solution. The primary supernatant was adjusted to pH 5–6 with acetic acid, freeze-dried, and kept at −50°C. The pellet containing bacterial cells was resuspended with 10 ml 0.1% acetic acid and heated in a boiling water bath for 20 min to extract toxin. This solution was applied to centrifugation at 13,000 rpm for 15 min, and the resulting supernatant was freeze-dried and stored at −20°C. In preparation for HPLC, samples were reconstituted with distilled water, passed through 0.22-μm Millipore filters, and loaded into SEP-PAK C_{18} cartridges (Waters Associates). Samples were then eluted rapidly with 0.1% acetic acid, collected, and applied to HPLC.

A quick and simple bioassay employing mouse neuroblastoma cell culture (ATCC No. CCL 131) was recently developed by Kogure *et al.* (1988) which can detect picogram levels of sodium channel blocking neurotoxins. The biochemical basis of the assay is that veratridine enhances sodium ion influx in neuroblastoma cells when Na^+-K^+ ATPase is inhibited by ouabain and that TTX is able to effectively block this enhanced influx. The cell culture bioassay uses this cancellation of effects, and the presence of toxin in assay samples is demonstrated by cultures that remain viable despite the presence of veratridine and ouabain. Using TTX as a standard, the calculation of cell death ratios allows for the quantitative estimation of sample toxicity as shown by Kogure *et al.* (1988).

To ascertain that the sodium channel blocking toxin accumulated in bacterial cultures was TTX, ion-paired reverse-phase HPLC was carried out on a JASCO Tri Rotar-VI HPLC system with a 6 × 300 mm silica ODS column (Senshu Scientific Co.) following methods described previously (Nagashima *et al.*, 1987). The mobile phase consisted of 0.05 *M* phosphate buffer (pH 7.0) with 1% methanol and 2 m*M* heptanesulfonic acid delivered isocratically. The eluent was mixed with an equal volume of 3 *N* NaOH at 100°C and fluorescence was monitored at 381 nm excitation and 505 nm emission on a Hitachi F1000 fluorescence spectrophotometer. Three microliters of TTX (Sigma) solution (0.1 mg/ml) was used as a standard. Although HPLC alone does not unequivocally prove the existence of TTX, it is a valid method for the confirmation of TTX in bacterial cultures given the results of Simidu *et al.* (1987), Tamplin *et al.* (1987), Yotsu *et al.* (1987), and sources cited therein.

Results

Bacterial colonies formed on all agar plates within a few days of incubation at 20°C. Swarming colonies were found on every plate regardless of the species of chaetognath or location of sample collection. On some plates this swarming colony dominated and no other colonies formed. These colonies were slightly arborescent, milky colored and were determined to be facultative anaerobes by the Hugh-Leifson test, designating them as members of the family Vibrionaceae. The majority of the other kinds of bacteria colonies tested negative for the Hugh-Leifson test and were excluded from the remaining testing procedures. Every *Vibrio* strain isolated from the heads of the chaetognaths *Flaccisagitta lyra, Parasagitta elegans, Zonosagitta nagae,* and *Eukrohnia hamata* was identified as *Vibrio alginolyticus* (Table I) following Baumann and Schubert (1984) and West and Colwell (1984).

Extracts taken from the batch cultures of all 15 *Vibrio* strains (Table I) effectively blocked the sodium channel as determined by the Kogure *et al.* (1988) cell culture bioassay. Within 24 h, extracellular toxin was present in the culture medium at concentrations ranging from 280 to 790 ng/ml (200 ng TTX equals one mouse unit) as estimated by cell culture bioassay. Ion-paired reverse-phase HPLC of both culture supernatant and bacterial cell extracts indicated that the responsible toxin was TTX (Fig. 1). Closely related TTX analogues have been shown to peak within several minutes after TTX using similar HPLC methods (Nagashima *et al.*, 1987; Yasumoto and Michishita, 1985), and these may also be present in the sample elution profile.

Discussion

Although Thuesen *et al.* (1988) found TTX in the heads of six species of chaetognaths, they did not determine the source of toxin production. The concentrations of extracellular TTX found in bacteria cultures indicates that *Vibrio alginolyticus* is capable of secreting TTX at substantial rates. This and the presence of *V. alginolyticus* in the heads of the arrowworms *Flaccisagitta lyra, Parasagitta elegans, Zonosagitta nagae,* and *Eukrohnia hamata* suggests that this bacterium is responsible for the production of the TTX in chaetognath venom. It is not yet known how many of the approximate 100 species of arrowworms use venom to capture prey, although the ecological diversity and morphological similarity of the

Table I

Characterization of the tetrodotoxin-producing bacteria of chaetognaths

Chaetognath	*Flaccisagitta lyra*	*Parasagitta elegans*	*Zonosagitta nagae*	*Eukrohnia hamata*	
Number of bacteria strains isolated	12	22	6	10	
Number of *Vibrio* strains (Hugh-Leifson test)	4	3	3	5	*Vibrio alginolyticus*
Chaetognath *Vibrio* characteristics					
Swarming	+	+	+	+	+
Growth at 40°C	+	+	+	+	+
Tolerance to 10% NaCl	+	+	+	+	+
Utilization of sucrose	+	+	+	+	+
Production of:					
Amylase	+	+	+	+	+
Gelatinase	+	+	+	+	+
Arginine dihydrolase	–	–	–	–	–
Acetoin (Voges-Proskauer)	+	+	+	+	+
Tetrodotoxin	+	+	+	+	+

known venomous species suggests that use of TTX venom may be very common throughout the phylum Chaetognatha (Thuesen *et al.*, 1988).

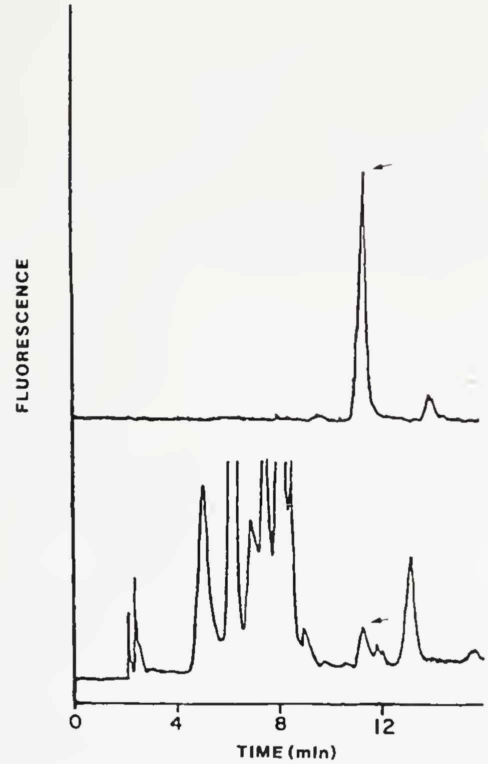

Figure 1. Ion-paired reverse phase HPLC elution profiles of tetrodotoxin standard (upper) and sample extract (lower) of the culture supernatant of *Vibrio alginolyticus*, which was isolated from the head of *Flaccisagitta lyra*. Some of the peaks following the TTX peak (arrows) within several minutes may possibly be related, less toxic tetrodotoxin analogues.

Of the six species of chaetognaths investigated previously (Thuesen *et al.*, 1988), *Parasagitta elegans* was found to have the highest toxicity: 320 pg of TTX per individual. The present study found *Vibrio alginolyticus* capable of secreting 280–790 pg of TTX in one microliter of culture medium within 24 h, figures that reasonably indicate that *V. alginolyticus* can replenish venom TTX over a necessary time scale. However, exact TTX secretion rates, numbers of *V. alginolyticus* inhabiting chaetognaths, and times required for replenishment of venom TTX following prey capture are not yet known.

The only other animal known to possess a TTX venom is the blue ringed octopus, *Hapalochlaena maculosa* (Scheumack *et al.*, 1978). TTX in the venom of *H. maculosa* is also apparently produced by bacteria (U. Simidu, pers. comm.). It has been suggested that *Vibrio alginolyticus* is the bacterium responsible for toxification of the tetraodontid fish, *Fugu vermicularis* (Noguchi *et al.*, 1987) as well as a toxic starfish, *Astropecten polyacanthus* (Narita *et al.*, 1987) and horseshoe crab, *Carcinoscorpius rotundicauda* (Kungsuwan *et al.*, in press). Our findings that *V. alginolyticus* can secrete TTX over a short time scale at relatively potent concentrations strengthens the argument for bacterial production of TTX in these animals. We consider *V. alginolyticus* to be the most apparent source of the TTX in chaetognath venom, albeit possible that venom TTX is produced by another as yet unidentified microorganism. However, it is remarkable that this identical TTX-producing bacterium has been found in association with such a wide array of marine animals which possess TTX.

Investigations of chaetognath-bacterial interactions are few. Unidentified bacteria have been reported as pathogens of chaetognaths both in the natural environment and in the laboratory (Nagasawa and Nemoto, 1984; Nagasawa *et al.*, 1984, 1985). Nair *et al.* (1988) investigated the bacterial flora of the neritic arrowworm *Aidanosagitta crassa* and found that *Vibrio* accounted for 12.7% of the bacteria associated with healthy individuals and 36.3% of those associated with weak and moribund specimens. These investigations were not developed further and identification of bacteria at the species level was not carried out. It is likely that *V. alginolyticus* accounted for some of the *Vibrio* species found by Nair *et al.* (1988), since all the *Vibrio* isolated in the present study were this species.

The exact location of *Vibrio alginolyticus* and the mechanism of accumulation of TTX in chaetognath heads is not yet known. Bieri *et al.* (1983) suggested that venom may be secreted from the vestibular papillae that lie beneath the tips of the posterior teeth, and this could be a sight of symbiosis. The vestibular pit, mouth, and gut are other possible locations of bacteria. The ultrastructural characteristics of bacterial associations has not yet been revealed in any of the animals that accumulate TTX, and it is not yet known if bacteria are enclosed within bacteriocytes or live within intercellular space. The role that TTX plays in the physiology of marine bacteria is still unknown, however its ability to stop the flow of sodium ions through cell membranes suggests that it may function in the regulation of ion exchange.

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3. ***Tables, footnotes, figure legends, etc.*** Authors should follow the style in a recent issue of *The Biological Bulletin* in preparing table headings, figure legends, and the like. Because of the high cost of setting tabular material in type, authors are asked to limit such material as much as possible. Tables, with their headings and footnotes, should be typed on separate sheets, numbered with consecutive Roman numerals, and placed after the Literature Cited. Figure legends should contain enough information to make the figure intelligible separate from the text. Legends should be typed double spaced, with consecutive Arabic numbers, on a separate sheet at the end of the paper. Footnotes should be limited to authors' current addresses, acknowledgments or contribution numbers, and explanation of unusual abbreviations. All such footnotes should appear on the title page. Footnotes are not normally permitted in the body of the text.

4. ***A condensed title*** or running head of no more than 35 letters and spaces should appear at the top of the title page.

5. ***Literature cited.*** In the text, literature should be cited by the Harvard system, with papers by more than two authors cited as Jones *et al.*, 1980. Personal communications and material in preparation or in press should be cited in the text only, with author's initials and institutions, unless the material has been formally accepted and a volume number can be supplied. The list of references following the text should be headed LITERATURE CITED, and must be typed double spaced on separate pages, conforming in punctuation and arrangement to the style of recent issues of *The Biological Bulletin*. Citations should include complete titles and inclusive pagination. Journal abbreviations should normally follow those of the U. S. A.

Standards Institute (USASI), as adopted by BIOLOGICAL ABSTRACTS and CHEMICAL ABSTRACTS, with the minor differences set out below. The most generally useful list of biological journal titles is that published each year by BIOLOGICAL ABSTRACTS (BIOSIS List of Serials; the most recent issue). Foreign authors, and others who are accustomed to using THE WORLD LIST OF SCIENTIFIC PERIODICALS, may find a booklet published by the Biological Council of the U.K. (obtainable from the Institute of Biology, 41 Queen's Gate, London, S.W.7, England, U.K.) useful, since it sets out the WORLD LIST abbreviations for most biological journals with notes of the USASI abbreviations where these differ. CHEMICAL ABSTRACTS publishes quarterly supplements of additional abbreviations. The following points of reference style for THE BIOLOGICAL BULLETIN differ from USASI (or modified WORLD LIST) usage:

A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some BIOLOGICAL ABSTRACTS listings).

H. A few well-known international journals in their preferred forms rather than WORLD LIST or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond., Science, N.Y.; Evolution, Lancaster, Pa.*)

6. ***Reprints, charges.*** Authors will be charged the excess over $100 of the total of (a) $30 for each printed page beyond 15, (b) $30 for each table, (c) $15 for each formula more complex than a single line with simple subscripts or superscripts, and (d) $15 for each figure, with figures on a single plate all considered one figure and parts of a single figure on separate sheets considered separate figures. Reprints may be ordered at time of publication and normally will be delivered about two to three months after the issue date. Authors (or delegates for foreign authors) will receive page proofs of articles shortly before publication. They will be charged the current cost of printers' time for corrections to these (other than corrections of printers' or editors' errors).

Editor's Note

With this issue, Charles B. Metz will retire from the editorship of *The Biological Bulletin* after a successful 10-year tenure. Marine Biological Laboratory President and Director Harlyn O. Halvorson is pleased to announce that Michael J. Greenberg has agreed to serve a five-year term as Editor-in-Chief of the journal. Dr. Greenberg is currently the director of the University of Florida's Whitney Laboratory in St. Augustine, Florida. Please note that all manuscript correspondence should still be sent to the Managing Editor at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543.

—P.L.C.

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Reference: *Biol. Bull.* **176:** 195–217. (June, 1989)

Calcium, An Overview—1989

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Abstract. An overview of calcium is presented including introduction, pre-history, chronology of the research recorded in the literature, discussion, summary, recent references, literature cited, acknowledgments, and appendix.

Elemental calcium began with the Earth's formation. Calcium was used for utilitarian purposes in B.C. times. In the 12th and 13th centuries A.D., calcium oxide was formed by roasting limestone to form calcium carbonate. A test for calcium was found in the 17th century, and "stones" were observed in humans (see appendix). In the 19th century, calcium was isolated and chemically identified by electrolysis, and later in that century calcium was found to be needed in a physiological solution similar to the ionic content of blood. In the 20th century it was found that, in the absence of calcium, living cells pulled away from one another. Anesthesia was produced by massive injection of magnesium salts into a mammal—conciousness could be restored by the addition of calcium, which neutralized the magnesium. Finally, calcium out of control in necrosis has an invasive action. Calcium antagonists and their mode of action were described in 1986.

Introduction

While a university fellow and research assistant to the late Dr. L. V. Heilbrunn, I developed a deep interest in calcium—an interest that has culminated in this manuscript after 48 summers at the Marine Biological Laboratory in Woods Hole, Massachusetts. This is my tribute to Dr. Heilbrunn for his research and profound vision of the importance of calcium in biological systems.

In 1983 the role of calcium dominated my conversations with Associate Professor Dr. Lincoln Ford of the University of Chicago. He urged me to write this account of the history of calcium.

The calcium ion is of fundamental importance in all biological systems. It participates in numerous biochemical and physiological processes. It is an intracellular regulator and also functions in extracellular mechanisms. For example, calcium is involved in neurotransmission, muscle contraction, blood clotting, cell adhesion, cell movement, permeability, and the surface precipitation reaction. It is also a second messenger, a major component of bone and teeth, and is important in agriculture and nutrition (Fig. 1).

Since calcium plays such a central and compelling role in all of basic biology, many books would have to be written in order to discuss all the phases and functions of calcium. Literature on the history of calcium dates back to 2600 B.C; the first practical problem—"stones"—was recorded in 1683 A.D. (see appendix).

Publications on calcium's importance, however, were sporadic and drew little attention until the Heilbrunn and Wiercinski paper (1948) describing microinjection of calcium into isolated muscle fibers. At that time, A. V. Hill questioned the slow diffusion of calcium into the cell. Niedergerke (1955) gave further impetus and brought attention to calcium with intracellular microinjection and electrolytic transport.

Since then, the interest in, and study of, calcium has increased dramatically. Approximately 22,000 papers have been published between 1966 and 1987 on the generation of concepts, hypotheses, mechanisms, models, postulates, and theories concerning calcium (Table I).

Pre-History

The overview of calcium begins with the origin of the sun, the earth, and other planets formed by the condensation of interstellar gases, particles, and elements brought together by Newtonian forces and by thermal,

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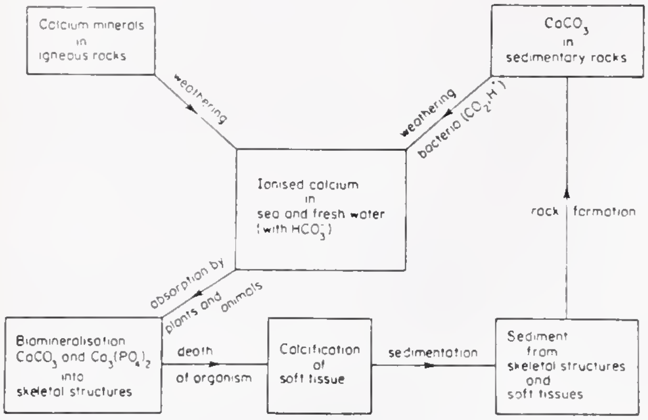
Calcium minerals in igneous rocks
weathering
$CaCO_3$ in sedimentary rocks
weathering
bacteria (CO_2, H^+)
Ionised calcium in sea and fresh water (with HCO_3^-)
rock formation
absorption by plants and animals
Biomineralisation $CaCO_3$ and $Ca_3(PO_4)_2$ into skeletal structures
death of organism
Calcification of soft tissue
sedimentation
Sediment from skeletal structures and soft tissues

Figure 1. Calcium phosphate cycle in the biosphere. The relationship between plant and animal life to the land and sea cycle. (Reprinted with permission; from Nichols and Wasserman, 1971).

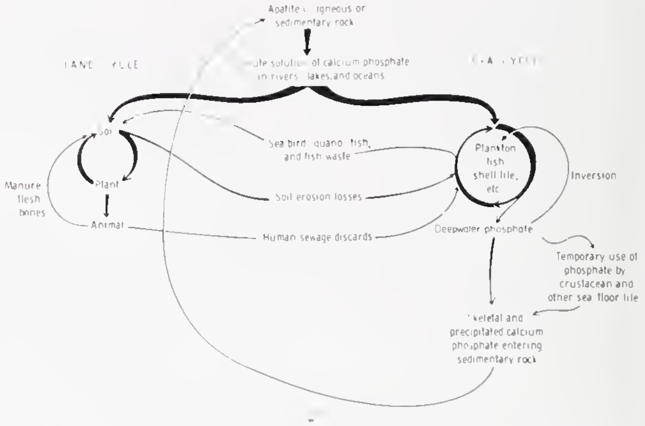
Apatite in igneous or sedimentary rock
LAND CYCLE
Dilute solution of calcium phosphate in rivers, lakes, and oceans
SEA CYCLE
Soil
Plant
Animal
Manure flesh bones
Sea bird guano, fish, and fish waste
Soil erosion losses
Human sewage discards
Plankton fish shell life, etc
Inversion
Deepwater phosphate
Temporary use of phosphate by crustacean and other sea floor life
Skeletal and precipitated calcium phosphate entering sedimentary rock

Figure 2. The biogeochemistry of calcium. The precipitates of calcium phosphate and carbonate are the major inorganic constituents of skeletal structures. In combination with collagen, rigid and strong structures are formed. (Reprinted with permission; from Campbell, 1983.)

electrostatic, and magnetic attractions at the time of the sun's formation. The earth went into orbit around the sun. Rock formed as the earth was pulled together by gravitational attraction. Consequently the planet grew in size. One major constituent of the new planet was calcium.

Water formed from hydrogen and oxygen. This filled the valleys and ocean basins of the earth. Water washed the rocks of the earth, leaching out elements including calcium into the ocean. Calcium is still being deposited into the oceans as sedimentary $CaCO_3$ from combination with carbon dioxide (Fig. 1). See Figure 2 for the biogeochemistry of calcium.

Ancient calcium

"The calcium carbonate of the rivers would supply this substance in 5×10^5 years. This has been deposited as sedimentary limestone. Since the formation of the oceans, water must have carried to the dwellers of the sea not less than 3×10^{17} tons . . . of calcium carbonate, which they have utilized as structural material" (Henderson, 1913).

Table I

Computer search (1966–1987) on concepts about calcium in the literature

	Agricola 1979–87	Biosis 1981–87	Life Sciences 1978–87	Medline 1966–87
Concepts	12	167	127	577
Hypotheses	13	651	611	1,880
Mechanisms	49	3,393	2,392	7,728
Models	16	207	260	3,446
Postulates	0	4	3	16
Theories	1	16	15	65
TOTAL	91	4,438	3,408	13,712

Grand total: 21,649 papers over 21 years.

If we take 5×10^5 years and divide it into 3×10^{17} tons of calcium carbonate, we would have 6×10^{11} tons per year. Forty percent of calcium carbonate is calcium. This is $6 \times 10^{11} \times 0.4$, which equals 2.4×10^{11} tons of calcium per year. This was deposited as sedimentary limestone on ocean floors.

Ancient calcium, with other ions, has been added to the oceans for 100 million years. Data from Williams (1971; *loc. cit.*, Sillan) shows that calcium is the third ion in abundance after sodium and magnesium. The fourth most abundant ion is potassium. These ions are essential for living systems.

Chronology

Early observations

From earliest times, man recognized that something was necessary in the structure and function of bones, teeth, and blood. That it was calcium became known much later. The history of calcium spans the last five centuries. A list of the functions of the calcium ion in time, as well as the scientists involved in determining those functions, follows.

B.C.

2600, Egypt: Gypsum ($CaSO_4$) mortar was used to create a seal between limestone blocks to preserve a boat in a sealed pit (Monastersky, 1987).

1336, Egypt: Alabaster, gypsum, used for vases, figurines, unguent jars, and chests.

Egypt: Plaster used to cover bandages used for wrapping mummies; the masks were made of plaster (Van Rens, 1987).

China: Limestone and mortar used in Guilin, a 2000-year-old city built during the Han Dynasty (Fang, 1987, pers. comm.).

Romans: Made plaster casts of body parts. Decorations in buildings and mortar (Van Rens, 1987).

1500, Ebers Papyrus: No mention of calcium. Sea salts were added to a mixture of herbs, made into a ball and inserted into the anus as a cure for diarrhea (Dawson, 1935).

400, Hippocrates: Used sounds to explore urinary bladder for calculi (in Berdoe, 1893).

A.D.: 12th–13th century

Limeburners: Reduction of limestone ($CaCO_3$) to lime (CaO).

17th century

1666, Boyle: White precipitate for calcium salts with sulfuric acid. Used as a test.

1666, Boyle: Aquafortis, Aquaregia, and Oyl of Vitriol. Used on various substances.

1667, Fairfax: Mr. Goodrich, surgeon at Bury St. Edmund's, found 96 small stones in the bladder of a lad. He also found a stone almost as large as a newborn child's head, and much of that shape.

1672, Lister: A stone was cut from under a man's tongue.

1683, 1693, Slare: A stone the size of a kidney was found. Stones also found in the vessels of the pelvis (see appendix).

18th century

1753, Black: Efficacy of limewater in dissolving urinary calculi, "the stone."

1786, Muller: "Sarcode droplets" on a *Colpoda* protozoan.

1794: Arabs used gypsum plaster casts (Van Rens, 1987).

1797, Wollaston: Chemical analysis of renal calculi.

19th century

1800, Volta: Voltaic pile battery.

1808, Davy: Calcium was isolated by electrolysis and the decomposition of alkalis.

1808, Davy: Metals obtained from alkaline earths.

1829, Dobereiner: Classified elements (see Discussion).

1835, 1841, Dujardin: Film formation around droplets emerging from a protozoan in calcium solution.

1846, Von Mohl: The name of "protoplasm" applied to the fluid within the cell.

1852, Muthijens: A new method of applying plaster bandages in the care of broken bones (Van Rens, 1987).

1857, Haeckel: Crustacean nerve was pressed, forms of "droplets, thread, and granules" came out of the nerve.

1882, 1886, 1890, Ringer: Physiological salt solution for cell function as the external electrolyte.

1885, Ringer and Buxton: Distilled water is actually harmful to protoplasm. Minute quantities of salts, calcium, sodium, potassium obviate the destruction.

1886, 1892a,b, Gruber: Cutting experiments, *Stentor* and structure, *Infusoria,* all in calcium containing media.

1888, Fabre-Domergue: Film formation around droplets of protoplasm pushed out of cells.

1890, Ringer: Added calcium chloride to distilled water and life was sustained longer than in sodium and potassium salt solutions. Tap water worked because of the calcium in it, whereas distilled water did not.

1892, Loew: Calcium counteracts magnesium when the magnesium concentration is too high in plants.

1892, Loew: Nucleus contains calcium mainly in the chromatin and the nucleolus.

1894, Locke: Calcium necessary for the transmission of excitatory nerve impulses from nerve to muscle. A nerve-muscle preparation in calcium-free solution, the muscle failed to respond when nerve was stimulated. Addition of small amount of calcium restored excitation by nerve. This response depended on the presence of calcium in the solution.

1894, Ringer and Sainsbury: "Remarkable power with a minute dose of lime salt, *e.g.*, lime phosphate possesses in maintaining the integrity of tissues." "Whilst a very minute quantity of a lime salt serves to be adequate to the maintenance of this vitality (of the worm *Tubifex*), the quantity may be increased enormously without producing any very definite results."

1896, Biedermann: 0.6% NaCl was found to be better with Na_2HPO_4 and Na_2CO_3 (Vol. 1, p. 105).

20th century

1900, Herbst: Cells tend to separate in the absence of calcium.

1902, Kolsch: Film formation around droplets of protoplasm in calcium media.

1902, Loeb: Pure calcium solution is toxic to *Fundulus* eggs. Isotonic solutions of calcium are toxic to cells and organisms adapted to living in the presence of relatively high concentrations of salts.

1903, Stiles; 1912, Trendelenburg; 1920, Alday-Redonnet; 1926, Gellhorn: Smooth muscle is more permeable to calcium than skeletal muscle. A prompt shortening occurs.

1904, Overton: Shortening occurs in uninjured muscle. Calcium necessary for transmission of impulses across the nerve synapse.

1905, 1908, Meltzer and Auer: Injected magnesium salts into the blood stream of a vertebrate. A sufficiently high concentration produced a state of anesthesia. When calcium was injected, the animal became sensitive and awakened.

1902, Loeb: Calcium and other ions in irritability and contraction.

1907, Benecke: *Spirogyra* not injured by calcium alone.

1908, Daniel: The destructiveness of distilled water has been attributed to its low content of inorganic salts. Distilled water of low conductivity and of physical purity was obtained.

1909, Mendel and Benedict: Excretion of calcium and interrelationship to inorganic compounds.

1909, Osterhout: K and Na act antagonistically.

1910, Meyer; 1926, Gray; 1941, Robertson: Calcium important in holding cells together.

1911, Mines: Exclusion of calcium from Ringer's solution caused uncoupling of the action potential of heart muscle from contraction. This was the first evidence of the role of calcium in coupling excitation and contraction.

1913, Spaeth: K and Na act antagonistically. K is seven times more potent than Na.

1916, Peck and Meltzer: Surgical operations were performed with magnesium anesthesia en masse (see 1905–1908).

1918, Loewi, a, b: Calcium is necessary in the digitalis effect on the heart (p. 93). Calcium prevents digitalis standstill in the heart.

1920, Alday-Redonnet: Adrenalin and calcium.

1920, Hober; 1932, Gillespie and Thornton: All types of muscle fail to contract in the absence of calcium.

1921, Loewi: "Vagus-stoff" from vagus nerve was a fundamental discovery. In the course of events it was observed that calcium plays an important role in the secretion of acetylcholine (ACH) that is "vagus-stoff."

1922, Burridge: "The paralyzing action of cocaine must be associated with some form of deprivation of the effected tissues of their calcium."

1922, Tate and Clark: Calcium chloride produces contraction of rabbit and cat uteri; relaxation of in guinea pig and rat uteri.

1923, Robison: Advance concerning conversion of blood calcium into the insoluble calcium of bone.

1925, 1931, Loew; 1926, 1928, Pringsheim: All animals from amoeba to man and all plants except some lower algae and fungi require calcium. A few yeasts and molds do not require calcium.

1926, Chambers and Reznikoff: Studied the actions of monovalent and divalent ions on amoebae using immersion experiments and microinjection.

1927, Doflein and Reichenow: Cutting experiments of Protozoa in calcium solution.

1927, Bouckaert and Colle: Magnitude of muscle contraction is a function of the amount of calcium present.

1927, Fitting: Calcium antagonizes the effect of poisons.

1927, Gley and Bouckaert; 1928, Gley; 1928, Houssay and Molinelli; 1929, Titajew and Unik: When calcium is lacking, nerve fibers, especially fibers of the autonomic system, fail to respond to stimulation.

1927, Heilbrunn: "Surface precipitation reaction." When *Stentor* was crushed in a calcium medium, a new membrane formed around the extruded cell protoplasm.

1928, Guissani; 1939, Beutner: Convulsions in dogs by injection of cocaine can be prevented by simultaneous (injection) action of calcium chloride.

1928, Kapeller and Kutschera-Aichbergen: Calcium is necessary for heart muscle function.

1928, Lawaczek: Adrenaline seems to release calcium from the bound to the free state.

1928, Pollack: By microinjection of calcium, precipitating agents showed the relationship between intracellular calcium and cell function such as movement.

1928, Rubenstein: Diagram for antagonism of cations (p. 408, Heilbrunn, 3rd edition). K and Na are mutually antagonistic. Ca and Mg antagonize Na but are synergistic.

1928, Simon and Szeloczey: Cocaine causes release of calcium from tissues.

1929, McCutcheon and Lucke: Both calcium and magnesium induced permeability compared to sodium and potassium.

1930, Chambers and Howland: Injections of $CaCl_2$, produce a dense granular coagulum in amoeba.

1930, Heilbrunn: High calcium concentration prevents protoplasmic clotting. Calcium is necessary for the surface precipitation reaction; higher concentrations of calcium ion have the same effect as other ions in retarding or inhibiting it.

1930, Pauling and Goudsmit: The level scheme of calcium, the metastable state.

1931, Camac: Medical anatomy from Imhoteps to Harvey.

1931, Kisch: An ion that is antagonistic to a second ion at one concentration may act with it at another concentration. According to Kisch, this relation exists between Mg and Ca.

1931 Kisch; 1934, Lasnitzki: Calcium lack may cause a decrease in the rate of respiration.

1932, Gillespie and Thornton: Isolated bronchial muscle of guinea pigs is sensitive to changes as low as 1 mgm 0/0 ionic concentration of calcium as measured by the response to histamine (p. 425).

1932, Heilbrunn: Magnesium and potassium anesthesia in amoeba. The colloid chemistry of protoplasm.

1934, Bacq and Rosenbleuth; 1936, Hukuda and Moriya: Heart muscle stops in a contracted state (systole) with calcium.

1938, Gaede: The working of the alkaline salts.

Antagonism of cations

1932, Costello: Calcium is a necessary requisite for the surface precipitation reaction in marine eggs.

1932, Heilbrunn: Simplest type of antagonist depends on the valence of the cation (p. 467). In sea urchin eggs, both the Mg and Ca tend to prevent the toxic effects of monovalent cations Na and K.

1932, Hermann: Calcium is necessary for the production of adrenaline.

1933, Breder: Marine fish can withstand fresh water when it contains a small amount of calcium.

1933, Ellis: Calcium and the resistance of *Nereis* to brackish water.

1934, Hastings *et al.:* The behavior of calcium in the presence of citrate, holding calcium in a non-ionizable form, in regard to the state of calcium in the living organism.

1934, Heilbrunn: The effect of anesthetics on the surface precipitation reaction. At low calcium concentration, ether inhibits the surface precipitation reaction in *Arbacia* eggs.

1934, McLean and Hastings: Frog heart used to test quantitatively the presence of definite amounts of calcium ion. Perhaps the best physiological example of reversible binding of calcium ion to serum proteins.

1934, Peters *et al.:* When calcium is added to normal uninjured mammalian heart tissue, it may cause an increase in the rate of respiration. At a given initial fiber length, the mechanical energy of the heart is increased under the influence of calcium.

1935, Buchanan: Very dilute solution of calcium salts protect planaria from the effects of distilled water.

1935, Dawson: Healing pagan and Christian (see 1500 B.C., Ebers Papyrus). Egyptian physicians used seawater in a suppository.

1935, Huf: Concept of active transport.

1936, Keil and Sichel: Injection of small quatities of $CaCl_2$ into isolated frog muscle M/200 caused violent but reversible shortening. (No data on quantity given.)

1936, Wiercinski and Child: Differential susceptibility of living organisms to supersonic vibration in calcium media.

1937, Ellis: Calcium plays a role in the exchange of water and electrolytes in *Nereis diversicolor.*

1937, Marcozzi; 1939, Mast and Pace: All commercial preparations of magnesium salts contain calcium.

1937, von Euler: Calcium chloride in skeletal and heart muscle studies in concentrations of 0.1 mol/l is necessary.

1937, von Euler; 1939, Greville: Respiration of minced muscle is decreased by calcium.

1937, Heilbrunn and Wilbur: Sodium citrate in seawater inhibited the germinal vescicle breakdown of *Nereis limbata* eggs induced by agents such as ultraviolet light or hypertonic potassium chloride in seawater. The normal response of the egg was restored by adding Ca^{2+}.

1937, Mazia: Release of Ca^{2+} was found in homogenates of fertilized *Arbacia punctulata* eggs.

1938, Ashkenaz: Narcotizing action of magnesium for whole muscle and single fibers can be antagonized by adding calcium ions to the medium. Recovery is best in Ringer's solution or in Ca-Mg-Ringer's solution containing four times the calcium content of ordinary Ringer's.

1938, Eggleton *et al.:* Calcium considered in 13 citations on all phases of heart action (frog) (see references).

1938, Goreczky and Ludany: In man, injection of excess calcium into the blood stream lowers the basal metabolic rate.

1938, Mast and Fowler: Sodium, potassium, and calcium ions in relation to volume changes in *Amoeba proteus.*

1939, Alexander *et al.:* Ca cephalinate is relatively insoluble compared with Na or K cepalinate in a liquid/liquid interface.

1939, Graham and Sichel: Local application of $CaCl_2$ to the surface of isolated skeletal muscle fiber caused reversible shortening of the muscle substance.

1939, Henry and Kon: In all comparisons, milk Ca was more available than the Ca of $CaHPO_4$, $2H_2O$.

1939, Mollison: Calcium deficiency lowers rate of respiration in plants—especially of the tops.

1939, Wiercinski: The effect of supersonic vibration on reconstitution and head frequency in *Euplanaria dorotocephala* in calcium medium.

1940, Guttman: Calcium maintained the resting membrane potential from other effects such as high potassium.

1940, Harvey and MacIntosh: In perfusion fluid lacking calcium, the release of acetylcholine was diminished or abolished. The effect of calcium was on the release reaction.

1941, Axelrod *et al.:* Calcium had a marked effect on the cytochrome-succinic-dehydrogenase system.

1941, Glick: Cholinesterase activity increased by calcium.

1941, Heilbrunn and Ashkenaz: The irritability of the isolated (cut) fiber is dependent on the calcium ion.

1941, Heilbrunn and Ashkenaz: Calcium rather than sodium (Overton, 1904) appears to be the ion essential for muscular contraction as demonstrated with isolated muscle fibers.

1941, McDonald and Kunitz: Calcium markedly increased the proteolytic activity of pancreatic juice. The effect results from the activation of trypsinogen to trypsin.

1941, Wiercinski: Intracellular pH determination in the centrifuged hyaloplasm of the *Arbacia* egg in seawater.

1942, Bailey: Calcium is a potent activator of isolated myosin ATPase.

1942, Bailey: Calcium influences the activity of lipases. Calcium activates the enzymatic activity of myosin.

1942, Elvehjem: Mechanism in enzyme system may be an indirect one (see Swingle *et al.*, 1942).

1942, Swingle *et al.:* Calcium stimulates activity of succinoxidase, which oxidizes succinic acid.

1943, Heilbrunn: Calcium, magnesium, and other cations have very marked effects on protoplasm. To some extent these actions may be due to their action on enzyme systems. (p. 201)

1943, Heilbrunn: There is also an interesting analogy in the behavior of protoplasm toward calcium. (p. 466)

1943, Heilbrunn: Magnesium narcosis and reversal to a conscious rabbit by injection of calcium. (p. 461)

1943, Heilbrunn: Researchers used improper concentrations of magnesium and calcium salts in their experiments; these were not isotonic. (p. 464)

1943, Heilbrunn: When a frog nerve is placed in isotonic calcium chloride solution, a wave of death proceeds from the cut end. This wave travels at a rate of a third of a millimeter per minute (unpub. experiments of M.A. Robbins, see p. 466, footnote).

1943, Heilbrunn: Some effects ascribed to calcium are due to the hypertonicity of the calcium solution.

1943, Heilbrunn: "In sea urchin eggs a trace of calcium will protect the cells from the toxic action of sodium, and a further increase in calcium concentration has but little obvious effect" (p. 463).

1943, Heilbrunn: "The fact that these and other drugs affect the cell by way of its calcium is but another indication of the importance of calcium in the vital machinery" (p. 467).

1943, Heilbrunn: Cocaine, like many other anesthetics, can also at times show a stimulating action (Ch. 36, 37). Both as an anesthetic and a stimulant, cocaine is antagonized by calcium. (p. 467)

1943, Heilbrunn (from Ringer and Buxton, 1885): Cilia of a freshwater clam gill could beat for eight days in a pure calcium solution.

1943, Heilbrunn (from Ringer, 1890): Frog eggs can develop normally for days in a calcium phosphate solution.

1944, Wiercinski: Intracellular pH determination of hyaline protoplasm in centrifuged *Arbacia* eggs in seawater. Cytoplasmic pH in *Amoeba* proteus in calcium media.

1945, Hober: In the series of Na, K, Ca, and Mg, Ca is high in hydration.

1947, Heilbrunn and Wiercinski: Solutions of sodium, potassium, magnesium, and calcium chlorides were microinjected into isolated frog muscle fibers in calcium-free media. Calcium ion, in high dilution (M/5000) caused an immediate and pronounced shortening. Injectant was 10% of the muscle fiber volume. The fibers were dissected in calcium-free media.

1950, Terry; 1954, Gross; 1968, Bianchi: Cytoplasmic inclusions swell and rapidly dissolve in low calcium concentrations.

1952, Castillo and Stark: End plate potential may reach about three times its normal size due to high calcium.

1952, Engback: Magnesium and calcium ions affect neuromuscular transmission. Calcium ion acts as an activator.

1952, Wiercinski: Microinjection of M/5000 $CaCl_2$ into the isolated frog muscle fiber induces protoplasmic shortening at alkaline and acidic pHs.

1954, Gross: Calcium can disrupt cytoplasmic granules.

Pharmacology

1918a,b, Loewi; 1937, Gold and Kwit: Digitalis and similar compounds act on the vertebrate heart only if calcium is present.

1942, Otis; 1964, Hammond: Relation of digitalis and adrenaline effects to calcium in oyster heart.

1951, Heilbrunn: Calcium is not only involved in bones and teeth, but also in the vital mechanisms of soft protoplasm.

1956, Heilbrunn: Coagulative properties of calcium on lower organisms and the relation to blood clotting.

1956, Heilbrunn: Coagulative properties and stimulating properties of calcium.

1958, 1966, Katz: Calcium functions in the amphibian neuromuscular junction to release acetylcholine during depolarization by nerve impulses.

1958, Shanes: Calcium maintained nerve conduction in spite of the influence of other agents to alter the resting potential.

1965, Pease *et al.:* Electron microscopic localization of calcium uptake in glycerol extracted psoas muscle fibers.

1965, Ratnoff: Hemorrhagic disorders: coagulation defects and calcium (in Beeson and McDermott, 1975).

1966, Kliner and Orten: Lactose and vitamin D increase calcium and strontium absorption.

1967, Federation Meetings: pH and calcium in muscle.

1968, Douglas: Calcium is a general mediator of secretory mechanisms (see Rubin, 1982, recent references).

1968, Katz and Miledi: Facilitation is larger during the second impulse, in accordance with the calcium hypothesis.

1968, Langer: Calcium and ion fluxes in myocardial contractility.

1969, Carriker and Smith: Comparative calcibiocavitology.

1969, Carriker *et al.:* Calcium carbonate substrates.

1969, Jahn and Bovie: Cellular cytoplasmic movements in cells. Protoplasmic motility involved in an actomyosin protein complex triggered by calcium to split high energy phosphates.

1970, Ford and Podolsky: Regenerative calcium release in muscle cells.

1970, Podolsky and Teicholz: Comparison of calcium contraction kinetics in skinned and intact muscle fibers.

1971, Williams: The metals of life. Importance of calcium.

1973, Goodenough and Stenger: The name calcium comes from *calix*, the Roman word for lime.

1973, Swanson *et al.:* Saliva, calcium, and potassium concentrations in the detection of digitalis toxicity.

1974, Esmon and Jackson: The function of fragment 2 during presence of factor V in blood clotting.

1974, Rubin: Speculation on calcium actin. (Rubin, 1982, recent references)

Enzymes

1905a,b,c,d, Delezenne: Dilute solutions of calcium salts favored activation of the enzyme; more concentrated solutions had the opposite effect. Analogy is made to blood. Dilute calcium solutions favor clotting, more concentrated solutions prevent it.

1937, Eagle; 1940, Ferguson; 1940, Brinkhous; 1940, Wohlisch: Modern theories of blood clotting emphasize the important role that a proteolytic enzyme plays in the process involving calcium.

1966/67, Fleckenstein *et al.:* Coined the term "calcium antagonist" based on observations of verapamil and prenylamine to interfere with the mediator function of Ca^{++} in the excitation-contraction coupling of heart muscle.

1969, Godfraind and Kaba: The term "calcium antagonist" was suggested because of the action of cinnarazine and chlorpromazine to inhibit the entry of calcium into depolarized smooth muscle cells.

1975, Arnold: Calcium causes contraction of the cleavage furrow in squid embryo cells. Embryos in calcium-free seawater will not cleave; an exaggerated cleavage of the dividing cells occurs in 2× calcium seawater.

1975, Palade: Secretion, intracellular aspects of protein secretion.

1976, Considine (ed.): Van Nostrand's Scientific Encyclopedia, 5th ed., "Calcium."

1976, Leaf and Cotran: Calcium, magnesium, and phosphate in pathorenal physiology.

1977, Suttie and Jackson: Prothrombin.

1977, Fleckenstein: Calcium antagonism in heart and smooth muscle. Calcium channels blocked by organic calcium antagonists *e.g.*, verapamil and nephedipine, oxyverapamil (D-600), and prenylamine.

1978, Kretsinger: Calmodulin.

1979, Kretsinger: Calcium in neurobiology. A general theory of its function and application. Neurobiology Manual, MBL.

1979: Symposia Soc. Exp. Biol. N. XXXIII, pp. 165–192. Conclusion: calcium ion plays a nearly universal role in all cell activities. Ca ion and cAMP are second messengers.

1980, Cheung: Calcium binding proteins as calcium receptors have been extended to a broad range of cellular reactions and processes. Calmodulin plays a pivotal role in cellular regulation.

1980, Ebashi: Szent-Györgyi's actomyosin-ATP-K system, which did not require calcium for activation, suppressed the calcium concept. Also, Heilbrunn did not appreciate the role of ATP.

1980: *Harvard Medical School Health Letter*, Vol. 5, No. 7, p. 5. The final common pathway for all triggers that initiate spasm may be an increase in the calcium flow into the smooth muscle cells lining the coronary arteries.

1981, Slater: Calmodulin may help explain the mechanism by which calcium regulates vital intracellular activities.

1982, Anghileri and Anghileri: Biological systems and calcium.

1982, Chafouleas *et al.:* Cell division—calmodulin and the cell cycle.

1982a,b, Jaffe: Possibility of methylation of calcium receptors and the genome to induce reversion is an earlier embryonic state for carcinogenesis (p. 306).

1982, Nuccitelli and Deamer: When pH is decreased in crayfish slow muscle fibers, the delayed outward K^+ current conductance decreases, allowing the inward Ca^{2+} current to become regenerative, producing all-or-none action potentials in these normally relatively inexcitable cells. (p. 435)

1982, Rubin: *Calcium and Cellular Secretion.*

1982, Thomas: *Techniques in Calcium Research.*

1983, Durrier and Meijler: Calcium paradox, a destruction of the ultra- and microstructure of the myocardium, in ischaemic or perfusion damage.

1983, Campbell: Calcium and cell movement (pp. 206–256). Endocytosis and exocytosis: uptake and release of substances from cells (pp. 305–361). Intracellular Ca^{2+} may play a key role in mediating the effects of primary stimuli on exocytosis, endocytosis, fluid secretion, and possibly steroidogenesis. The rationalization of these phenomena and their evolutionary significance has to be made (p. 361).

1983, Jaffe: Sources of calcium in egg activation.

1983, Morton: An annotated checklist of text illustrating the history of medicine. Urinary calculi (pp. 572–589).

1983, Spiro: Calcium signal transmitted through the interactions with target enzymes and structural proteins.

1983, Tsien: Calcium channels in cardiac muscle are hormone responsive.

1983: Calcium Theme, 67th Annual Meeting of the Federation of American Societies for Experimental Biology and Guest Societies. Chicago, IL. April 10–15, 1983.

1983: *Federation Proceedings.* Calcium, phosphorus, and related elements. Nutrition.

1983: *MBL Newsletter,* "Calcium and the Man."

1984, Brown: Functional properties of calcium channels.

1984, Katz *et al.:* The data reviewed suggests that altered calcium homostasis may have some focal role in the etiology of the pathogenesis of cystic fibrosis.

1984, Medlars II: N.L.M.'s Interactive Retrieval Service, July 9, 1984. Citations printed back to 1966 for calcium.

1984, Poole-Wilson *et al.:* Calcium out of control in ischaemic heart muscle.

1984, Shiner and Solaro: The Hill coefficient for the Ca^{2+}-activation of striated muscle contraction.

1984, Somlyo: The cellular site of calcium regulation is in the endoplasmic reticulum.

1985a,b, Berridge: Secondary messengers serve as signals in the cell. Inositol trisphosphate induces the cell to mobilize still another messenger, Ca^{2+}. Calcium binds to a family of proteins, including calmodulin and troponin, to activate a protein kinase.

1985, Carafoli and Penniston: The calcium signal mediates a broad array of cellular functions. Protein pumps and other structures turn it on and off.

1985, Inesi: Vesicular fragments of the SR membrane provide a highly favorable system for detailed studies on the mechanism of Ca transport and coupled enzyme catalysis.

1985, Krebs: Intracellular calcium is the messenger responsible for the subsequent activation of calcium-dependent protein kinases and phosphoprotein phosphatases.

1985, Martin: See chapter 11, "Calcium, phosphorus, and calciferols," and chapter 12, "Parathyroid hormones, calcitonin, and other regulators of calcium and phosphorus metabolism."

1985, Rubin *et al.: Calcium in Biological Systems.*

1985, Schuel (in Metz and Monroy, 1985): Function of egg cortical granules. Calcium stabilizes structure and may be source of intracellular calcium released at fertilization.

1985, Silinsky: Calcium ion is considered a full antagonist to evoke ACh (actylcholine) release. Strontium ions are partial agonists. Magnesium ions are competitive inhibitors to evoke ACh release. A calcium binding protein associated with the secretory apparatus is suggested.

1985, Wichterman: Many references to calcium in the biology of *Paramecium.*

1986, Abdel-Latif: Regulation of cellular function by neurotransmitters, hormones, and a variety of regulatory and growth-promoting factors.

1986, Evans *et al.:* Corn root cap with calcium containing agar block restores the root's gravotropism. The mobile calcium in the cap is necessary for the root to respond to gravity.

1986, Gross: "The truth about calcium."

1986, Little *et al.:* Alterations in calcium conductance may be involved in the ethanol withdrawal syndrome. This result offers possibilities for the development of non-sedative therapeutic treatment.

1986, Putney: Evidence of a capacitative model for non-excitable cells such as exocrine glands and liver. In excitable cells, a receptor activation (modulation) of voltage-regulated Ca^{2+} channels is suggested.

1986, Thomsen: Plasma physics breaks stones.

1987, Augustine *et al.:* Calcium action in synaptic transmitter release.

1987, Bignold: General cell motility—membrane ratchet model and ameboid movement.

1987, Crone: Endothelial derived relaxing factor (EDRF). The endothelial system of the plasmalemmal invagination participates in the control of cytosolic calcium concentration.

1987, Newland: Blood coagulation: a review.

1987, Stolz and Bereiter-Han: Sequestration of iontophoretically injected Ca^{2+} into monolayer cultured living tadpole heart cells.

1988, Stolz and Bereiter-Hahn: Calcium sensitivity of microtubule changes.

1988, Stolz and Bereiter-Hahn: Increase in cytosolic calcium and formation of F actin aggregates.

1988, R. Silver (pers. comm.): One atom of calcium was observed in the cytosol of the sanddollar egg with the video microscope.

Discussion

Importance of calcium

Calcium has appeared in the literature in one form or another since 2400 B.C. It is now known to be important in the external movement of cells, tissues, and organisms, in the intracellular movement of organelles, and in the formation of by-products released from cells.

Total understanding of the intra- and extracellular functional aspects of calcium must be considered using a multifaceted approach. Several authors have recently examined aspects of calcium's role in biological systems. Rubin *et al.* (1985) considered the modern historical and biological aspects of calcium action dating back to the time of Sydney Ringer (1882–1894). Schwartz and Azar (1981) described intracellular regulation and transport leading to calcium's control of physiological processes, and Campbell (1983) described intracellular calcium as the universal regulator.

We now know that eukaryotic cells maintain free Ca^{2+} within the cytosol at a concentration between 10^{-7} and 10^{-8} M (Kretsinger, 1980). In muscle fibers, calcium is stored in the sarcoplasmic reticulum (Costantin *et al.*, 1965). Calcium's role in the formation of calculi in the kidney, gall bladder, and joints, as well as in the formation and structure of bones and teeth, are well documented.

Calcium's biological roles

Calcium plays a number of important biological roles (Rubin, 1974, 1982; Schwarz and Azar, 1981; Anghileri and Anghileri, 1982; Campbell, 1983; Ebashi, 1984; Carafoli and Penniston, 1985; Rubin *et al.*, 1985).

Structurally, calcium is involved with second messengers, neurotransmitters, biomembranes, mechanical stress and muscle proteins, and cell-to-cell communication. Chemically, it is involved in the release of hormones. It is required for the maximum activation and regulation of enzymes and proteins, and it also serves as an intracellular regulator in association with calmodulin and hormone cyclic nucleotides. Calcium helps to maintain osmotic balance within cells by modulating cations, anions, and water permeability. It is also involved, in a variety of ways, in oxidation-reduction. Calcium's roles in tissue culture cells are also diverse: it is involved in adhesion, spreading, locomotion, cytokinesis, shape changes, and the closing of intracellular junctions. Finally, calcium plays important roles in cell motility (ruffling, blebbing, locomotion, displacement of mass, migration, and chemotaxis of leucocytes).

Isolation of calcium

Davy began work on electrolysis after Volta's battery appeared in 1800. He isolated components of alkali salts dissolved in water. In 1808 he announced the isolation of potassium and sodium as mutants. Using similar methods, he isolated barium, magnesium, calcium, and strontium (Leicester, 1956).

Dobereiner (1829) then classified elements and found "triads" with similar properties. Calcium, strontium, and barium was one such triad, the atomic weight of the middle member being approximately the arithmetic mean of the weights of the other two elements.

Level scheme of calcium

The lowest energy state of calcium occurs when the two outer electrons are both in the 4s orbit. When the single level occurs, the triplet is non-existent. In calcium's excited state, only one of the two outer electrons is excited, the other remains in the 4s state. The triplet levels are the so-called "metastable states." Via collision with other atoms, a calcium ion can drop to its "normal" state or the atom can absorb radiation to a state of higher excitation. It can then drop to the lowest level with the emission of radiation (Russell, 1927; Pauling and Goudsmit, 1930).

Plaster of Paris

The ancient Egyptians and Romans applied techniques of using plaster of Paris as it is known today. The plaster bandage is hard setting and made of such components as gypsum ($CaSO_4$), starch, albumen, lead-vinegar, and spirits of camphor (van Rens, 1987).

Gypsum was used by the Arabs in 1794 in the treatment of fractures. They placed the fractured limb in a mold and poured gypsum over the broken extremity (van Rens, 1987).

In the 19th century, the use of plaster bandages was not easily accepted in Europe. The Thomas-urgoden splint was favored in England for quite some time.

The "stone"

Calculi (stones) can occur in any hollow organ in the body in which its fluid content is stagnant. Stones have been known since ancient times. Descriptions of them have been found in ancient Summarian (pre-Babylonian) and early Egyptian writings. Dissection of mummies more than 5000 years old have revealed calculi, and in 900 A.D., the Arabians devised an operation for the removal of bladder stones (Benton, 1972).

In the 17th century, Boyle (1666) noted the formation of calcium when a calcite precipitate formed in sulfuric acid. Later, Fairfax (1667) and Slare (1683, 1693) reported "stones" in humans (see appendix). Black (1753) tried using limewater to dissolve urinary calculi. In 1797, Wollaston found renal calculi consisting of a mixture of uric acid, calcium, magnesium, ammonium phosphate, and calcium oxalate. Nearly two centuries later, Thomsen (1986) used laser beams to disintegrate kidney stones. In 1989, extracorporeal shock wave lithotripsy shatters kidney and biliary stones. The body is immersed in water (*Health Scene*, 1989).

Hypotheses generation

A number of concepts, hypotheses, mechanisms, models, postulates, and theories have been generated regarding calcium since the 1st century A.D.

Venantius Fortunatus (600 A.D.) used the Latin word “protoplasma,” meaning “first created thing, protoplast” (in Greek meaning “a moulded thing, figure, or form”). Müller (1786) described “droplets” in the protozoan *Colpoda*. This was an extrusion of the cell content into the surrounding water.

Dujardin (1835) postulated the concept of a “sarcode,” an elementary substance composing the protozoan. Dujardin (1841) observed film formation of cell cytoplasm emerging from *Leucophrys striata*, a protozoan parasite found in the earthworm. Von Mohl (1846) named the sarcode “protoplasm.” Huxley (1868) considered protoplasm “the physical basis of life.”

Fabre-Domerque (1888) and Kolsch (1902) observed film formation around droplets of extruded protoplasm from a cell.

Heilbrunn (1930) postulated the “surface precipitation reaction.” When the cell cortex is broken, the emerging protoplasm forms a membrane to seal the break in the presence of calcium ion. When cells are immersed in calcium-free media, the protoplasm forms vacuoles and flows outward from the injured cell.

Calcium is associated with a receptor protein in cells. In the “bleeders disease” where fibrinogen is inadequate, the blood does not clot because calcium, although present, has no fibrinogen to produce the fibrin required for blood clotting.

It is evident that Ca needs an “acceptor protein” in the cell. Heilbrunn (1928) suggested ovalbumen for the *Arbacia* egg; Kretsinger (1978), a protein in the cytosol; and Chance (1965), formation of an intermediate Ca_2 ($\sim$1) with the displacement of two protons—perhaps a metastable calcium as suggested by Pauling and Goudsmit (1930). Cheung (1980) suggested calmodulin, and others suggested leotonin, protein kinase, and protein kinase C.

In summary:

Heilbrunn (1930) postulated the role of calcium in the cell, and the “surface precipitation reaction.”

Heilbrunn (1951) wrote that calcium plays many roles in the vital life processes such as cell division, aging, surface precipitation reaction, clotting of blood and cells, muscle contraction, and in a variety of ailments—from acne to the hardening of arteries.

Gross (1954) discussed the mechanisms for the yolk lysis reaction.

Chance (1965) wrote about the accumulation of calcium.

Fleckenstein (1977) discussed calcium antagonists such as verapamil.

Kretsinger (1979) wrote on the theory and evolution of calcium in neurobiology.

Cheung (1980) described calmodulin as playing a pivotal role on calcium.

Ebashi (1980) wrote that in 1960 there was considerable doubt about the “calcium hypothesis.”

Campbell (1983) asked: does a stimulus activate 50% of the maximum possible “switched on” cells, or do all the cells give 50% of their individual response?

Putney (1986) reviewed hypotheses on the mechanism of calcium entry into the cell. He also wrote about the calcium pump.

Peterson (1987) discussed larger molecules, such as proteins and enveloping smaller molecules.

Physiological solutions

Ringer (1882, 1886, 1890) recognized the need for a physiological salt solution similar to the ionic content of blood. When calcium chloride was added to distilled water, life was sustained longer than in sodium and potassium solutions.

Ringer and Sainsbury (1894) found that minute doses of lime salt were necessary for the survival of the worm *Tubifex*.

Locke (1893, 1894) found that calcium was necessary for the transmission of nerve impulses from nerve to muscle.

Loeb (1906) wrote that life phenomena, and especially irritability, depend “on the presence in the tissues of a number of various metal proteids, or soaps (Na, Ca, K, and Mg) in definite proportions.” Na-salts cause rhythmical contractions in striated muscle only if the muscle cells contain Ca-ions. When there is a lack of Ca-ions in the tissues, the Na-ions are no longer able to cause rhythmical contractions. Because plants contain no muscle, there is no need for NaCl.

Anesthesia

Meltzer and Auer (1905–1908) observed anesthesia in a rabbit following injection of a solution of magnesium. Upon injection of a small amount of calcium solution, the rabbit awakened and was sensitive. Peck and Meltzer (1916) performed surgical operations in humans, using magnesium anesthesia. Dogs will also go into anesthesia with magnesium salts (Wiercinski, pers. obs.).

Active transport

Huff (1935) suggested the concept of "active transport." Huff recognized the relationship of cellular metabolism and the role of extracellular metabolites to be transported into the living cell and the transport of substances out of the cell. The essential criteria are based on the following conditions in the living cell: (1) a concentrated and hydrated product; (2) sensitivity to oxygen lack; (3) dependence on active metabolites; and (4) sensitivity to enzyme poisons.

LeFevre presented work on the human red cell. Prior to his 1955 monograph, he wrote about the penetration of glycerol (1946), active transfer (1947), certain nonelectrolytes (1948), active transport of monosaccharides (1954), and active transport through cell membranes (1954).

Bresnick and Schwartz (1968, pp. 305–362) discussed active transport: methods of study, theories, drugs, hormones, and membranes.

The active transport concept was accepted from 1955 onward. However, the term "active transport" was used in different ways by various authors (see Giese, 1973, p. 324). The relationship of sodium channels and calcium in active transport is currently (1987) being studied by Clay Armstrong at the Marine Biological Laboratory (Woods Hole, Massachusetts).

Frog heart study

Clark (1938) describes the following in the frog heart:

(1) Duration of electrical and mechanical responses.
(2) Excess, refractory period of the heart and calcium.
(3) Heart's content of calcium.
(4) Hypodynamic heart and calcium.
(5) Lack, carbohydrate balance of heart with calcium.
(6) Lack, electrical response of heart and calcium.
(7) Lack, electrical response and metabolism.
(8) Lack, heart arrest and phosphagen with calcium.
(9) Lack, heart metabolism and action of calcium.
(10) Lack, mechanical response of heart and calcium.
(11) Loss of calcium in the heart on perfusion.
(12) Plasma and content of calcium.
(13) Sympathetic stimulation and liberation of calcium.

Electrical properties

Living cells have an "electrical property." Calcium reacts with the isolated muscle fiber, as Niedergerke (1955) demonstrated with calcium and electrolytic transport in local contraction and partial relaxation. The calcium ions activate a link in the contractile cycle. An electrical stimulus causes contraction. Presumably, when it is subthreshold, contraction is not elicited.

Microinjection of freshly dissected muscle fibers

It is recognized as a primary principle in the study of living organisms that the "living state" is of the utmost importance.

Hober (1920) and Gillespie and Thornton (1932) found that all types of muscle lose the ability to contract in the absence of calcium.

Keil and Sichel (1936) microinjected an aqueous solution of M/200 $CaCl_2$ and observed reversible contraction. No quantity was stated.

Graham and Sichel (1939) applied $CaCl_2$ to a small portion of an isolated muscle fiber surface and observed a marked reversible shortening of the "muscle substance."

Heilbrunn and Wiercinski (1947), in an extensive work to prevent contamination of solutions, used quartz distilled water from previously distilled water to prepare the solution to be microinjected. Quartz micropipettes were prepared for microinjection. These were "flamed" and reused. Pyrex micropipettes were used only once. Calcium ion at M/5000 caused a 50% "shortening" of the muscle fiber as compared to sodium and potassium ions in isotonic concentrations. Magnesium ion caused an extension of the muscle fiber (about 10% of the fiber volume).

Niedergerke (1955) microinjected muscle fibers with calcium and electrolytic transport and saw contraction in a reversible state.

Caldwell and Walster (1963) injected caffeine into crab muscle fibers. A contraction (contracture) was followed by a relaxation. The caffeine released the calcium from sarcoplasmic reticulum.

Mechanism of muscle contraction

Mines (1911) gave the first evidence for the role of calcium between excitation and contraction in muscle.

Fanburg and Gergely (1965) reported that the mechanism for the relaxation and contraction of skeletal muscle may operate in heart muscle. Calcium accumulation for skeletal and cardiac cells is optimal when ATP, magnesium, and oxalate are present in the *in vitro* preparations of sarcoplasmic reticulum and mitochondria.

Mechanisms of cooperativity

Cell components must cooperate to maintain cell function. Calcium binding to cell organelles and molecules depends on concentration and affinity for proteins in the population of Na^+, K^+, Mg^{2+}, Cl^-, SO_2^{-2}, PO_4^{-3}, etc. According to Pauling and Goudsmit (1929), calcium exists in a metastable state. This would involve electrons in cellular reactions (Peterson, 1987). Larger molecular mechanisms in cells can join with smaller molecules to perform a variety of chemical functions in biological systems.

The most important mechanism of structure and function is cooperativity. Malphighi's discovery of the capillaries at least 200 years ago has recently been researched by Crone (1987) using the passage of solutions through capillary pores. Calcium may be an operative factor in the endothelial cells of the "endothelium derived relaxing factor" (EDRF).

Accumulation of calcium (based on Bianchi, 1968)

Chance (1965) postulated that accumulation of calcium can occur in the following steps (steps 5 and 6 are alternative mechanisms):

(1) Passive diffusion of calcium through the outer membrane of mitochondria (in the cytosol) to binding sites on the inner membrane;

(2) Formation of an intermediate of $Ca_2(\sim 1)$ with the displacement of two protons;

(3) Transport of the energy-rich intermediate complex across the inner wall of the mitochondria;

(4) Binding of calcium to a phospholipid component of the inner membrane leading to the release of the components of the intermediate complex and the stimulation of respiration;

(5) Formation of hydroxyapatite or calcium phosphate crystals, thereby removing calcium from the mitochondrial membrane wall and allowing further re-accumulation of calcium;

(6) Neutralization of the liberated protons by carboxylic acids and mitochondria swelling.

Trends

Loewi (1912, 1918a,b) found that calcium is necessary for the digitalis effect on the heart. He also performed the classical "vagus-stoff" experiment (1921). Later it was found that the active principle is acetylcholine. Calcium is necessary for this reaction.

Chambers *et al.* (1926, 1930, 1932) did studies using micromanipulation on amoeba, starfish, and *Arbacia* eggs.

Keil and Sichel (1936) observed the effect of injecting M/200 Ca^{2+} ion concentration in isolated muscle fibers. They did not determine injection volume.

Heilbrunn and Wiercinski (1948) microinjected Na^+, K^+, Mg^{2+}, and Ca^{2+} chlorides into isolated living adductor magnus muscle fibers of the frog. M/5000 $CaCl_2$ showed a 50% shortening of the muscle fiber. Isotonic $MgCl_2$ produced relaxation. The injection volume was 10% of the muscle fiber volume. This research brought Heilbrunn's insight about calcium into focus. In response to this work, Heilbrunn and Wiercinski opposed Szent-Györgyi's notion that potassium ion was the primary ion.

A. V. Hill and Albert Szent-Györgyi caused the "hush of neglect." Hill thought of diffusion in a solid cylinder, before the discovery of the sarcoplasmic reticulum. Szent-Györgyi applied ATP and K^+ ion in minced muscle to determine if this was the primary stimulus for muscle contraction. He made sure that the muscle preparation he used was "dead" in contrast to Heilbrunn and Wiercinski's work with freshly dissected muscle fibers in calcium-free media.

Stoltz and Bereiter-Hahn (1987, 1988) injected calcium by iontophoresis to observe sequestration in live endothelial cells in tissue cũlture.

Colloid chemistry of protoplasm

Heilbrunn (1928–1932) performed cutting experiments with *Stentor* and *Amoeba* and showed that the "naked protoplasm" quickly formed a film in calcium-containing solution. From these observations he postulated colloid chemistry as the basis of protoplasmic activity. He believed that protoplasmic activity is transmitted from one part of a cell to another or from one cell to another. The reaction has three stages: (a) free calcium is liberated, (b) calcium unites with other materials to form a thrombin-like substance which, in the sea urchin, is named ovothrombin, and (c) a reaction occurs between this thrombin-like substance and another substance to form a precipitation product. He also found that ovothrombin is surface active at the rate of 25 cm/s, and that this is a self-propagating reaction.

Surface precipitation reaction

Heilbrunn (1928–1932) also described the surface precipitation reaction. He noted that the reaction is retarded or inhibited by low temperature, that calcium is essential, and that pigment granules play a role in the sea urchin egg. He also found that calcium is released in the cell interior, that calcium reacts with pigment granules or a constituent named ovothrombin, and that ovothrombin reacts with substances (sea urchin egg) in the protoplasm—presumably a protein—to cause vacuole formation. He also discusses the action of acids and alkalies.

Neurobiology: theory of function and evolution

Kretzinger (1979) showed that many basic cellular mechanisms are common to all eukaryotic cells, are of common origin, and are related in evolution.

Campbell (1983) showed that calcium plays a role in nerve terminals, neurohormones, neuromuscular junctions, neurones, neurophysin, neurosecretions, neurotransmitters, and receptors.

Mandel and Eaton (1987) and Snutch *et al.* (1986) characterized voltage gated calcium channels in *Xenopus* oocytes after the injection of RNA obtained from electrically excitable tissues. Mandel and Eaton (1987) and Chad *et al.* (1986) also showed that phosphorylation maintains calcium channel activity in dialyzed molluscan neurons.

Five postulates have been suggested for the intracellular function in eukaryote cells.

(1) All resting eukaryotic cells maintain a concentration of free Ca^{2+} in the cytosol between 10^{-7} *M* and 10^{-8} *M*.

(2) The sole function of Ca^{2+} within the cytosol is to transmit information through binding to a specific receptor.

(3) The target of Ca^{2+}, functioning as a second messenger, is a protein in the cytosol.

(4) Calcium modulated proteins contain "E.F. hands" to maintain conformation (Krietzinger, 1978).

(5) Cells initially extruded calcium so they could use phosphate as their basic energy currency; $Ca_3(PO_4)_2$ is insoluble.

Comparison of concepts

Comparing Heilbrunn's (1928) work on the colloid chemistry of protoplasm with Kretzinger's (1979) work on the theory of the function and evolution of calcium, we find that each suggested five postulates about the function of calcium. Chance (1968) presented six postulates.

Homeostasis

Heilbrunn's general physiology textbooks (1938, 1943, 1952) do not mention homeostasis. However, he does discuss the topic in his 1956 text *The Dynamics of Living Protoplasm.*

Rasmussen and DeLuce (1963) reviewed calcium regulation extensively. They mention (p. 147) isolated mitochondria in calcium and phosphate exchange as a new approach to calcium.

Mathews *et al.* (1981) discuss the role of calcium in the organism as a reciprocal relationship between intracellular and extracellular functions. Currently, it is believed that the intracellular ionic concentration is 10^{-7} *M* and the extracellular is 10^{-3} *M*. A proper ionic intracellular calcium level is maintained by the mitochondria (p. 424). In bone mineralization, large amounts of calcium and phosphate are utilized.

The general physiological effects of calcium can activate or control basic physiological processes, such as irritability, contractility, conduction, secretion, mitosis, reproduction, and integration. The role of calcium in special nerve organs, other tissues, and cell types is being investigated.

McGrath *et al.* (1986) showed that active transport through the bone membrane appears to be involved in the regulation of calcium levels in the blood stream. The present development limits the possible active transport mechanisms for the mineral dissolution/deposition to an outward-directed pump for the hydrogen ion, an outward-directed pump for the phosphate ion, or an inward-directed pump for the hydrogen ion.

Stoltz and Bereiter-Hahn's (1987) observations support the assumption of two sequestration mechanisms: (1) a high affinity for Ca^{2+} capacity and a low affinity for Ca^{2+} (5×10^{-15} mols^{-1}) and (2) a low affinity for Ca^{2+} and a high capacity ($10–40 \times 10^{-15}$ mols^{-1}) for calcium accumulation. These mechanisms are saturable. The high affinity Ca^{2+} compartment is the endoplasmic reticulum, and the low affinity system is the mitochondrion. There is a five- to eight-fold higher Ca^{2+} sequestration capacity, and the velocity of sequestration is equal in both cell types (XTH2 and primary cells).

Calcium homeostasis

Claude Bernard (1878) proposed constancy of the internal environment of animals and plants. Subsequently, biologists have made a considerable effort to understand the regulation of this internal environment. The endocrine systems, the parathyroid gland, vitamin D-hormone, modern-day calcitonin, phosphate (ATP), and the mitochondria play roles in the integrated organic scheme.

Cannon (1932) postulated that homeostasis controls the internal environment of warm-blooded animals. Oxygen, pH, salts, food (glucose, amino acids, lipids), proteins, and blood concentrations are maintained within narrow limits. However, Richards (1953) criticised homeostasis. He claimed that in chronic disease, homeostasis does not prevail in all functional bodily processes.

Rasmussen and DeLuca (1963) discussed the parathyroid gland's role as an essential calcium control system in the mammal. In evolution the parathyroids first appeared in the amphibia. Teleosts all possess a calcified endoskeleton. Salmon, in their migratory passage from the sea to fresh water, achieved calcium homeostasis in spite of the dramatic fluctuations in the calcium content of the external environment. This happens without parathyroid glands. It is suggested that the role of the D vitamins in calcium homeostasis is older in evolution than the parathyroids. Calcium, phosphate, and vitamin D are essential in the diets of all higher animals.

In summary (pp. 164–165), the parathyroid hormone, vitamin D, and phosphate function in the cellular accumulation and transport of calcium in a variety of tissues. Vitamin D regulates the activity of calcium-specific systems in cellular and mitochondrial membranes. The ability of cells in epithelium to bring about the net transfer of ions, as distinct from ion accumulation, is related to structural characteristics and polarity.

Bianchi (1968) wrote that in mammals, calcium and phosphate are controlled by parathyroid hormones, vitamin D, and thyrocalcitonin in intracellular and extracellular function. Over-secretion of parathyroid results in osteitis fibrosa cystica, absence of vitamin D causes rickets in the young and osteomalacia in the adult, and overproduction of thyrocalcitonin may be related to osteoporosis. The underlying causes of disturbances in phosphate and calcium metabolism affect calcium transport across cell membranes and intracellular organelles.

Protein kinase and protein kinase C

A Ca^{2+} phospholipid-dependent protein kinase is recognized as a pivotal regulatory element in signal transduction, tumor promotion, cell regulation and rat brain cytosol (see below).

Protein kinase and protein kinase C are reported in *Methods in Enzymology* (1987) and by Mandel and Eaton (1987) (see also Chad *et al.*, 1987, for protein kinase, and Strong *et al.*, 1987, for protein kinase C).

Several methods have described protein kinases (phosphoproteins) from bovine heart, pig spleen, rat brain, rabbit renal cortex, and rabbit brain. When assessed with a 7–9% acrylamide gel, it is tentatively identified as having an "apparent" molecular weight of 80,000. "On 18% acrylamide gel, it migrates with an apparent molecular weight of about 120,000" (p. 419, *Methods in Enzymology*).

The calcium paradox and the multifaceted behavior of the calcium ion

Durrier and Meijler (1983) wrote that the calcium paradox is only one aspect of the multifaceted behavior of the calcium ion in all living material, but together in its role in the myocardial excitation complex it is a very important one. One of the most important spin-offs of the study of the effects of calcium on living tissue is the discovery of calcium antagonists (Fleckenstein, 1977).

As early as 1920 (see Heilbrunn, 1937, p. 348), there is a literature on "paradoxes." These include a potassium paradox, a calcium paradox, and a thermo paradox. Can these occur in the metastable state of calcium (Pauling and Goudsmit, 1930)?

The future of the calcium paradox model—for myocardial, ischaemic, and pre-perfusion damage—depends on the development of compounds that may protect the myocardium.

Anghileri and Anghileri (1982)

Volume I discusses the chemistry of calcium and gives both the theoretical and practical basis to interpret its role in the function of normal and pathological biological systems. The precipitation process of hydroxyapatite (HAP) as it relates to bone and tooth is reviewed in detail. Mathematical models for analyzing calcium kinetics are discussed.

Volume II reviews the role of calcium in membrane stability and the regulation of various membrane functions. It describes basic properties of the mechanisms for calcium inflow and outflow across the plasma membrane. It also assesses the results of investigations of plasma membrane, calcium movement in other tissues and summarizes the effects of hormones, neurotransmitters, other cell stimuli, and cell injury on these processes.

Volume III examines the role of calcium in the insulin release process, in taste and olfactory functions, and in dentinogenesis.

Calcium out of control

Poole-Wilson *et al.* (1984) wrote that calcium is invasive in myocardial cell necrosis following severe hypoxia or ischaemia. The mechanisms are: (1) lack of energy, loss of enzyme systems, (2) mechanical effects, damage of the structural matrix of the cell, and (3) membrane damage in the mitochondria and sarcolemma. The common pathway is cell necrosis, and calcium "overloads" the cell. A causal mechanism is the accumulation of calcium during myocardial hypoxia or ischaemia followed by reoxygenation or pre-perfusion related to the development of cell necrosis.

Pools

The idea of "pools" for calcium and other ions derives from the observations of food vacuoles in protozoa. Protozoa, such as *Amoeba*, take in food particles at the edge of the plasmalemma forming a food vacuole. Undigested particles are subsequently expelled by the food vacuole. *Paramecium* has a gullet for taking in food particles (endocytosis) and smaller protozoans. The undigested portion is taken into a contractile vacuole that is expelled (exocytosis) through the micropyle of the cuticle or cortex.

Heilbrunn (1937) suggested that bound calcium exists in the living cell. For example, calcium is bound in the cortex of the cell. Stimulation from a wide range of phenomena could elicit the release of calcium from the cortex—a pool. A 10 μm cell, as suggested by Brown (1984), is the typical size of cells in many tissues. The cortex could easily hold a pool of 3000 calcium ions. Permease could release this calcium to the cytosol. This would be in equilibrium with the calcium concentration of 10^{-7} to 10^{-8}.

How many calcium ions enter a living cell under physiological conditions? The calcium ion has an invasive action. When present in the cytosol in any amount greater than 10^{-7} to 10^{-8}, it causes breakdown of cellular function. Brown (1984) considered a spherical cell of 10 μm diameter. How many calcium ions would a cell this size require? Considering the concept of calcium pools in the cytosol of small cells (10 μm diameter), how many calcium ions would be present in a pool? The intracellular environment and anatomy of the cell should be considered. Brown considered 10^{-8} intracellular calcium activity: "The cell would contain about 3000 free Ca ions." Where are these Ca ions located in the cell? How many would be used during and following an "action potential"? What would be the number of Ca ions in a pool? Also, how many pools exist in the cytosol of a small cell? Have "pools" been detected as morphological entities? Porter (1961) found the endoplasmic reticulum, which extends from the cell membrane to the nucleus around the mitochondria and the Golgi apparatus.

At least three pools have been found in the cytosol of secretory cells (Campbell, 1983, p. 329). The cortex or cell membrane would be the site from which calcium ions can enter the cytosol to be stored in a pool (endocytosis). An event such as an action potential elicited by a stimulus would release calcium (exocytosis).

Campbell (1983, p. 224) writes that intracellular calcium can be altered by several mechanisms: (1) an effect on the permeability of the cell membrane, (2) An effect on the intracellular store, and (3) an effect on the calcium pump.

An acceptor protein with a chemotactic property would be a cyclic AMP protein kinase. *In vitro* studies have shown soluble and insoluble membrane-bound proteins.

There are calcium channels in the cortex as well as an endoplasmic reticulum in the cytoplasm. The molecular mechanism of intracellular calcium is as a regulator.

Calcium antagonists

A group of organic drugs block the entrance of calcium into living cells. The invasive property of calcium is to enter damaged cells. The surface precipitation reaction is a cell's response to injury.

In heart disease the heart cells could be damaged and the adverse action of calcium would cause the cell to disintegrate. But by preventing calcium from entering the cell, can it repair itself?

The classical experiment of crushing a cell under a coverslip on a microscope slide in a calcium environment produces the surface precipitation reaction. Cytosolic calcium keeps the nucleus and the cellular organelles (ribosomes, liposomes, mitochondria, etc.) in an intact membrane.

Fleckenstein *et al.* (1966/1967) coined the term "calcium antagonist." Godfraind and Kaba (1969) showed inhibition of calcium by cinnarizine and chlorpromazine of contraction in vascular smooth muscle. Nichols and Wasserman (1971) compared external and internal exchange of calcium in isolated cells. Rahwan and Witiak (1982) wrote that calcium entry into cells depends on the diversity of structure in multiple modes of action and sites of action.

Naylor and Dillon (1986) wrote:

"Ca antagonists are a novel group of drugs useful in management of a variety of cardiac disorders. They differ from one another in terms of their chemistry, tissue specificity and selectivity. As a group, however, they share the common property of slowing Ca^{2+} entry through voltage-activated, ion-selective channels. Some of them exhibit other properties, including that of interfering with Na^{+} transport. At least one of them, diltiazem, has an intracellular action. Specific high and low affinity binding sites have been identified for two of the major groups of Ca^{2+} antagonists, with the binding sites for verapamil and its derivatives being distinct from those which can be occupied by the dihydropyridines. The number (B_{max}) and affinity (K_D) of these binding sites changes under certain pathological conditions—including a reduction in ischaemia and in spontaneous hypertension, and increase in the latter, at present, only demonstrated for the dihydrpyridine binding sites. The sensitivity of a particular tissue to these drugs will depend upon a number of factors including the number of binding sites that are present, the contribution made by the Ca^{2+} entering tissue, and properties which are peculiar to a particular type of verapamil, they exhibit use-dependence."

Moser (1986) discusses calcium entry blockers in a historical perspective on the management of hypertension. These drugs lower blood pressure by reducing peripheral resistance through the vasodilation of the blood vessels and capillaries in blocking calcium that acts as a powerful vasoconstrictor.

Calcium homeostasis control

In their volume on calcium antagonists, Van houtte *et al.* (1988) include many papers to define the existence of various types of calcium channels. They write:

"The clinical perspective is represented by the most recent cardiovascular, renal, gynecological, endocrinological and neurological applications of calcium antagonism research; the ever widening circle of disorders to which calcium antagonists are being applied now include heart failure, hypertension, stroke, migraine headaches, epilepsy, vertigo, dysmenorrhea, and certain disfunctions of the kidney and liver."

Historical reviews and a broad survey of current research define new directions for future research. For example, work pointing toward the treatment of long-term diseases, particularly atherosclerosis, is discussed, and data are presented that indicate that some calcium antagonists enhance low-density lipoprotein uptake by increasing the number of low-density lipoprotein receptors. These data, which indicate that calcium antagonists could be active at the gene level and thus affect mRNA and protein synthesis, suggest entirely new approaches to drug research.

The possibility that calcium antagonists influence the central nervous system is explored. Researchers look forward to neural applications, such as protecting the brain against ischaemia and anoxia, that could prove as beneficial as the cardiovascular application.

The events triggered by exaggerated calcium influx and release clarifies how the harm caused by these events, which undoubtedly contribute to cell death, have prompted scientists to attempt the control of calcium homeostasis by reducing excessive calcium influx, facilitating intracellular calcium buffering, and counteracting calcium-evoked reactions.

Conceptual advances

Examination of current calcium literature indicates that there has been little, if any, inquiry into the molecular mechanisms that no doubt are involved in calcium at the atomic level. Dr. Robert Silver (Cornell University, pers. comm.) is observing one atom in the cytosol of the sand dollar egg using video microscopy techniques.

Conceptual advances in cellular recognition elements have recently been made involving cell receptors. Fourteen types of receptors that are specific for biological molecules in the cell have been recognized.

Putney (1986) and Peterson (1987) suggest a model for receptor-regulated calcium entry. Five previous hypotheses are reviewed. Putney's proposed model is termed "capacitative Ca^{2+} entry."

Clearly, all living cells are born with the inherent electrical property of life. Calcium does not "work" in the biological sense in non-living cells.

Unanswered questions

Much has been written regarding calcium's entry into and out of the living cell and its role in cell death. Recently developed techniques used by calcium researchers have opened up a vista of complex biochemical reactions.

Calcium is essential for life. It is present in plants and in the blood of animals, and it is used, as needed, in cellular functions. But what happens in the living cell? How many things can happen or be contained in one cell? Finally, what is the role of calcium in the living organism both on earth and in outer space?

Summary

After examining the extensive chronology about calcium, I have attempted to discuss the fundamental implications of calcium in a reciprocal role in maintaining the extracellular and intracellular functions in biological systems. Homeostasis is maintained in the living organism by numerous mechanisms, but this is not so in the diseased state.

The receptor protein calmodulin, protein kinase, protein kinase C, and a number of other proteins have been described. The calcium paradox found in cardiac muscle function remains. Why calcium?

The question, as yet, goes unanswered. No other substance is capable of maintaining, with the cooperativity of organismic systems, normal homeostasis while performing biological, building, and physiological functions.

In summary, this paper is merely an overview—a small piece of the much larger, complex, and ever-changing puzzle that is calcium.

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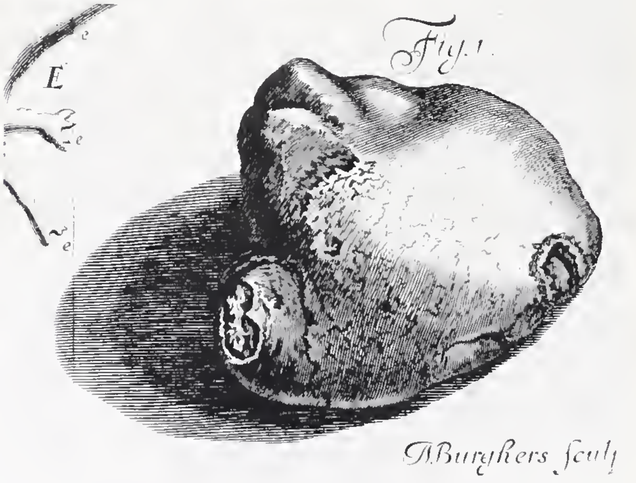
Appendix

A Poſtſcript *to the* Publiſher, *containing a ſhort account of two* Human Calculi *of unuſual form and bigneſs, from the ſame* F. S. M. D.

SIR,

I Here ſend you the Figure of a *Stone*, *Fig.* 1. of a prodigious *ſize* and as rare a *ſhape*, ſomewhat reſembling indeed the *Kidney*, for that was quite worn away, and this *ſton* fill'd up the *place*, it weigh'd indeed ſomewhat more when I took it out of the Body, than it does now, for it then weigh'd ſeven ounces and a half; there is no *Hiſtory* that relates any account of a *ſtone* generated in the *Kidney*, that does near parallel this. Without breaking it aſunder, I can find it does conſiſt of ſeveral *laminæ* laid over one another, as that of the *Bladder* does: I took the *Circumference*, and found it to meaſure ſeven Inches upon the round.

That taken out of the Body of the late *Duke* of *Norfolk's* Father, *Fig.* 2. was brought not long ſince to the *R. Society* by Sr. *Theodore de Vaux*, who gave me leave to ſend you the *Figure* of it, which you ſee is branched, and ſeems to have ſpread ſome branches into great *Veſſels*, whether *Arteries*, *Veins* or into the *Ureter* I cannot determine, tho theſe as well as the *Pelvis* ſeem to have been fill'd up by this great *ſtone*: yet this comes far ſhort of that before mention'd, ſince it *weighs* but four ounces and a half: a *ſtone* indeed of an incredible ſize to be found in the *Kidney*. The meaſure longwiſe from one extreme to the other made four Inches compleat: the extenſion of the *Branches* from one to the other meaſur'd croſwiſe or tranſverſly, 3 Inches and a half. This is deſervedly laid up in the *Rpoſitory* of the *Royal Society*, as a great but ſorrowful *Rarity*, having cauſed the death of ſo great a *Patron* of *Learning*.



From Slare (1693).

Reference: *Biol. Bull.* **176:** 218–228. (June, 1989)

Diel Variation in Prey Capture Behavior by the Corallimorpharian *Discosoma sanctithomae:* Mechanical and Chemical Activation of Feeding

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Abstract. The feeding biology of corallimorpharians is poorly understood. This paper describes an envelopment method of prey capture by a Caribbean species, *Discosoma* (= *Rhodactis*) *sanctithomae*, and further examines the stimuli that elicit envelopment and subsequent ingestion of prey. The corallimorpharians exhibited a diel pattern of expansion and contraction of the oral disc margin and tentacles. This was correlated with the density of zooxanthellae in these tissues. The tentacles expanded during the day to expose abundant zooxanthellae. The oral disc margin contained relatively few zooxanthellae and was contracted and turned down in a convex posture during the day. At night the margin was expanded and turned up to give the oral disc a bowl-shaped posture. This allowed effective execution of the envelopment response that was successful in the capture of crustaceans, polychaetes, and small fishes that were most abundant in the plankton at night. The stimuli that elicited this predatory behavior were both mechanical and chemical. Mechanical stimulation elicited envelopment at night but was not as effective during the day. Envelopment was also activated by the imino acid proline and the tripeptide reduced glutathione at low concentrations, and four other amino acids at high concentrations. Continued contraction and ingestion responses required chemical activation. Consistent ingestion responses were caused only by reduced glutathione.

Introduction

Cnidarians typically capture their prey with stinging and adhesive tentacles. However, members of the corallimorpharian family Discosomatidae are unusual in that their tentacles do not appear to be adapted for feeding (Den Hartog, 1980). Unlike other cnidarians they have few ectodermal nematocysts in their tentacles, and in contrast to all other taxa in the subclass Hexacorallia they completely lack spirocysts (an important taxonomic character of this group). Spirocysts are usually abundant in the tentacles of hexacorallians, and the adhesive character of these organelles is of major importance in catching and holding prey (Mariscal, 1984). Den Hartog (1980) states that the tentacles of the Discosomatidae are "of a quite unusual character, being non-retractile, non-motile, and practically devoid of musculature. It is difficult to assume that these tentacles are functional catching devices, and bearing in mind that Discosomatidae are invariably associated with extremely numerous zooxanthellae, it seems more likely that this group of Corallimorpharia is primarily, if not entirely, dependant on these associates (and therefore on light) for nutrition." He further notes that the feeding biology of this group is worthy of experimental study.

Indo-Pacific members of Discosomatidae (= Actinodiscidae) have been documented to feed by enveloping prey with their oral discs (Hamner and Dunn, 1980). The purpose of this paper is to describe a similar mode of feeding in a Caribbean member of this family, *Discosoma sanctithomae.* We also examine diel variations in the behavior and experimentally investigate the stimuli involved in eliciting prey capture and ingestion.

Materials and Methods

Field observations

The study was conducted at the Bermuda Biological Station for Research, Bermuda, during August, 1987,

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and two weeks in May, 1988. Surveys to determine the distribution and abundance of *D. sanctithomae* were done at many different sites in nearshore and offshore reef areas. Field observations and experiments were conducted at both Whalebone Bay and Coney Island (32° 40′ N, 64° 43′ W) during the day and night. Feeding behavior was examined by observing the corallimorpharians capture organisms that were attracted to dive lights during night dives. Day feeding observations were made by placing small amounts of live *Artemia* nauplii on the oral discs of the corallimorpharians with a medicine dropper.

Photographs were taken of four different clonal aggregations at Whalebone Bay during the day and night to document feeding behavior, determine maximum population densities, and examine diel variation in size and posture. The photographs were analyzed with a digitizer board (Bioquant digitizer software for IBM PC) to measure surface areas of different portions of the oral disc for patterns of contraction and expansion.

Collection and maintenance

A total of 170 specimens of *D. sanctithomae* were collected at Coney Island by chiselling off the dead coral to which they were attached. The animals were maintained in sea tables supplied with running seawater at temperatures of 22–24°C and a salinity of 36‰. The seatables were indoors where they received indirect ambient lighting from nearby windows as well as artificial lighting from overhead fluorescent lamps producing 15–20 $\mu E \cdot m^{-2} \cdot s^{-1}$. Observations and experiments were conducted under both ambient and artificial lighting during the day and fluorescent lighting at night. The animals received a variable light/dark cycle, being exposed to 12–18 h of light day^{-1} depending on the amount of time the fluorescent lights were on during the evening. The 50 individuals used in 1987 were not fed for the two week duration of the study, but the 120 animals used in 1988 were fed *Artemia* nauplii after one week. Some individuals were also used for observations of feeding on whole nereid polychaetes.

Sixty-three specimens of *D. sanctithomae* were collected using the above method at dawn on 17 May 1988. They were immediately placed in plastic bags and then preserved in 10% formalin. The animals were later removed from the coral to which they were attached and the bags were examined for contents that may have been egested during transfer. Each individual was examined under a dissecting microscope for coelenteric contents five days later. Specimens were cut open in a bowl of water and the contents of the coelenteron were removed and measured using an ocular micrometer.

Determination of zooxanthellae densities

The relative densities of zooxanthellae in different portions of the body wall of *D. sanctithomae* were measured using the method outlined by Sebens and DeReimer (1977). Animals were fixed in Bouins, sectioned in a cryostat, and then examined under a compound microscope at 400× magnification. The densities of zooxanthellae ($cells \cdot cm^{-3}$) in the discal tentacle and outer margin regions of the oral disc were measured.

Nematocyst discharge

Experiments were performed to determine if *D. sanctithomae* discharged nematocysts when contacted by a prey item or some other object. Glass coverslips and 1 cm^2 pieces of parafilm coated with dried *Artemia* homogenate were presented forcefully to the discal or marginal tentacles of the corallimorpharian. Densely packed *Artemia* nauplii in seawater were homogenized, centrifuged for approximately five min at maximum speed on an IEC model C-L tabletop centrifuge (*circa:* 1500 rpm) and the supernatant used in experiments. Similar nematocyst discharge tests were also done with the anemones *Condylactis gigantea* and *Aiptasia pallida* for comparison. The coverslips were stained in methylene blue then examined for the presence of nematocyts at 400× and 1000× magnifications on a compound light microscope.

Stimuli that elicit the feeding behavior

To determine the factors that elicit the envelopment response and subsequent feeding in *D. sanctithomae*, a series of mechanical and chemical stimuli were tested in both the field and laboratory. The effect of mechanical stimuli was tested in the field by gently passing a pasteur pipette through the discal tentacles of randomly selected specimens every 2 s and recording how many contacts were required to cause the envelopment response and complete closure of the oral disc margin. New pipettes were used for each specimen. This experiment was done during the day (13:00) and night (22:00).

The effect of chemical stimuli in eliciting the envelopment response was tested in the field during the day and night by directing a stream of solution (0.5 ml) from a syringe (5 ml with 18 guage needle) at the discal tentacles of a specimen from a distance of approximately 3 mm. Individuals separated by more than 1 m were used for each trial. The treatments used were *Artemia* homogenates (prepared as described above), 20 amino acids, 3 proline analogs, the tripeptide reduced glutathione (GSH), and S-methyl glutathione. Carbohydrates and lipids were not tested since they are not effective feeding activators in other cnidarians (Lenhoff and Lindstedt, 1974; Van-Praët, 1985; Sebens, 1987). All other chemicals were dissolved in glass fiber-filtered seawater collected from offshore in the Sargasso Sea. All solutions were used within 6 h to avoid auto-oxidation or loss of amino acids to bacteria. Stock solutions were prepared at 10^{-1} *M* and then serially diluted to the desired concentrations.

The behavior of the animals in response to the chemical stimuli was recorded as: (1) envelopment and complete closure of the opening to the cavity formed by the margins of the oral disc, (2) partial closure of the opening to the cavity formed by the margins of the oral disc, and (3) presence of mouth opening or extrusion of mesenterial filaments. Each animal was used in a maximum of three trials, and was tested only once in each 24-h period and at least 24 h after feeding.

The effect of mechanical and chemical stimuli in eliciting a continued feeding response was examined by first causing laboratory specimens to close in response to mechanical stimulation from a pipette. Then 0.5 ml of either plain seawater or a test solution was introduced with a pipette into the cavity formed by the margin of the oral disc. The behavior of the animals (amount of oral disc contraction) and the time taken for them to reopen and resume their normal posture was recorded.

To determine which chemicals were effective in causing both closure of the oral disc and ingestion, a piece of Whatman No. 1 filter paper (2 × 2 mm, with or without chemicals) was positioned 1 cm above the oral disc and dropped onto the discal tentacles. The presence or absence of closure and ingestion and the time taken for the oral disc to reopen and resume normal feeding posture was recorded. All filter papers were soaked in solutions of chemicals dissolved in deionized water and then dried for 12–48 h before being used in experiments. Filter papers that were soaked in chemicals and then dried gave more consistent responses than papers that were not dried before use. To determine if the corallimorpharians would ingest inert items when stimulated with chemicals in solutions, pieces of clean filter paper were dropped onto the oral disc (as described above) and then 0.5 ml of a solution of GSH or proline (10^{-2} *M*) was directed at the discal tentacles from a pipette to initiate closure.

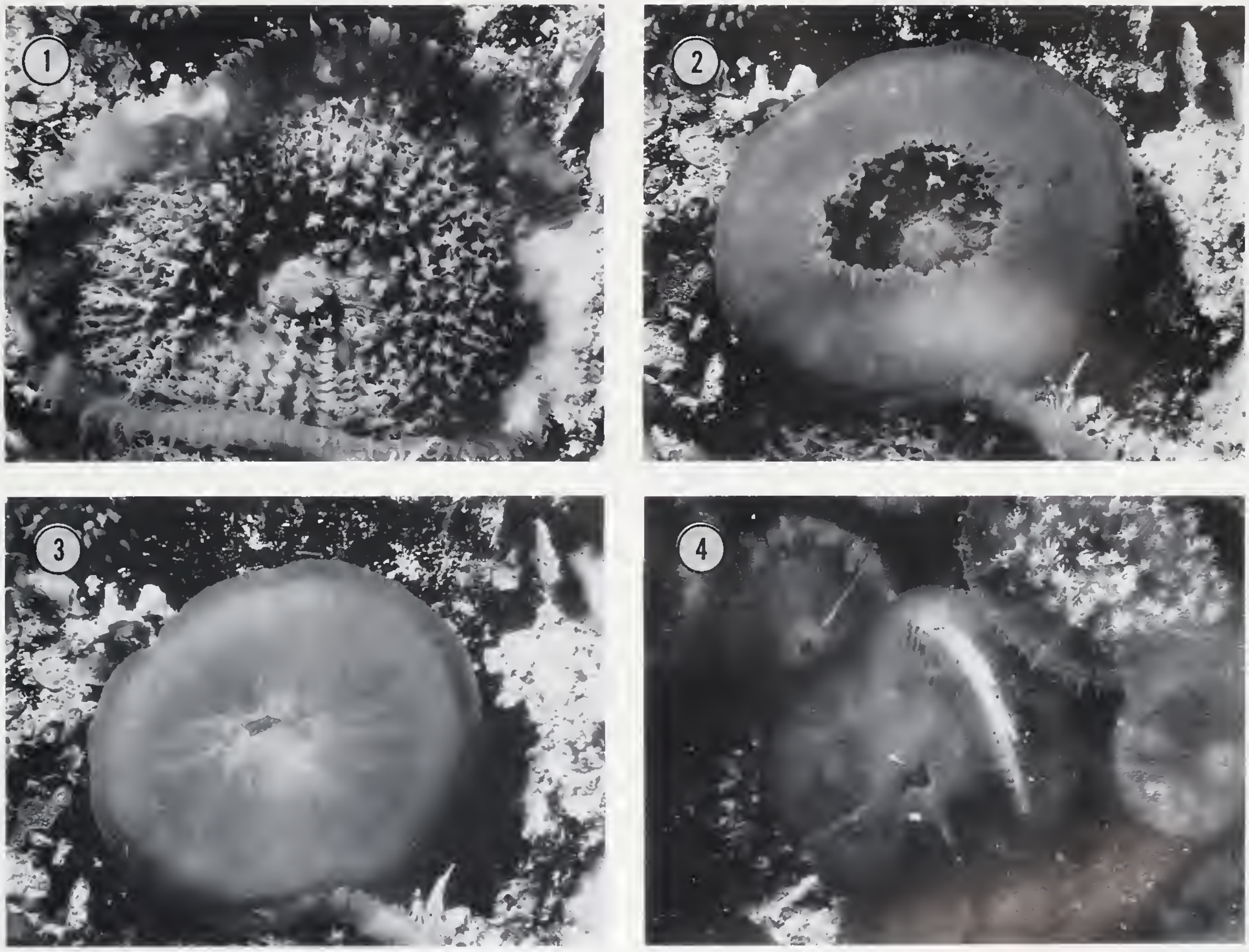
Results

Populations of *D. sanctithomae* were found on all reefs surveyed in Bermuda. This species reached maximum densities of 800 individuals$\cdot$m^{-2} at Coney Island and Whalebone Bay where it was one of the most abundant sessile invertebrates. Clonal aggregations were generally found on vertical surfaces in well-lighted areas from depths of 1 to 10 m. They were especially common in surge channels in all habitats.

Night diving observations of *D. sanctithomae* in August, 1987, revealed that they had a striking feeding behavior in response to being contacted by planktonic organisms (Figs. 1–4). Swarming polychaete epitokes, crustaceans, and small fish that contacted the corallimorpharians' oral disc caused the margins to be quickly pulled up and in (~1–3 s after contact) to form a cavity in which the prey was enveloped. This sequence of behaviors is very similar to that described by Hamner and Dunn (1980) for species of corallimorpharians found in the Indo-Pacific. After prey contact, the radial muscles of the oral disc contract to pull up the margin of the oral disc (Fig. 2) and then the circular muscles of the margin contract to close the opening to the cavity above the mouth (Fig. 3).

Captured prey continued swimming around inside the cavity for a few minutes until continued contraction of the margin reduced the amount of space and trapped the prey against the oral disc tentacles (Fig. 4). There was no adhesion of the prey to the discal tentacles or evidence of nematocyst discharge to cause the prey to be anaesthetized in the captures that were observed. However, since it was difficult to observe the events occurring inside the cavity through the tissue of the oral disc margin there may have been some stinging of the prey by the actinopharynx or extruded mesenterial filaments during ingestion. In laboratory tests no nematocyts were discharged onto coverslips or pieces of parafilm coated in *Artemia* homogenate presented to the discal tentacles, but homotrichs (*sensu:* Den Hartog, 1980) were detected on pieces of *Artemia*-coated parafilm presented to the marginal tentacles. The nematocyts in the marginal tentacles may prevent prey from escaping through the opening above the mouth until complete closure has taken place.

After prey capture the mouth of *D. sanctithomae* opened widely and the actinopharynx became distended. Laboratory observations of feeding showed that large prey (*e.g.*, nereid polychaetes) were moved to the mouth by contraction of the oral disc toward the mouth and by subsequent distension of the actinopharynx toward the prey item. Small prey such as *Artemia* were transported into the mouth by ciliary movement on the actinopharynx. It took approximately 45 min to 1.5 h for *D. sanctithomae* to ingest nereid polychaetes (measuring approximately 30 × 5 × 3 mm) and then reopen and resume a normal feeding posture. Undigested remains of prey were egested 24 to 48 h after ingestion. Of 63 field specimens collected at dawn, only two individuals had any prey items in their coelenterons. One individual contained a gammarid amphipod (2.3 × 0.7 × 0.5 mm) and the other had an unidentified crustacean appendage (5.0 × 1.2 × 1.2 mm). Other cnidarians are known to digest their prey within a few hours after capture, with no recognizable items remaining after two to six hours (Porter, 1974; Sebens and Koehl, 1984). Our analysis is probably

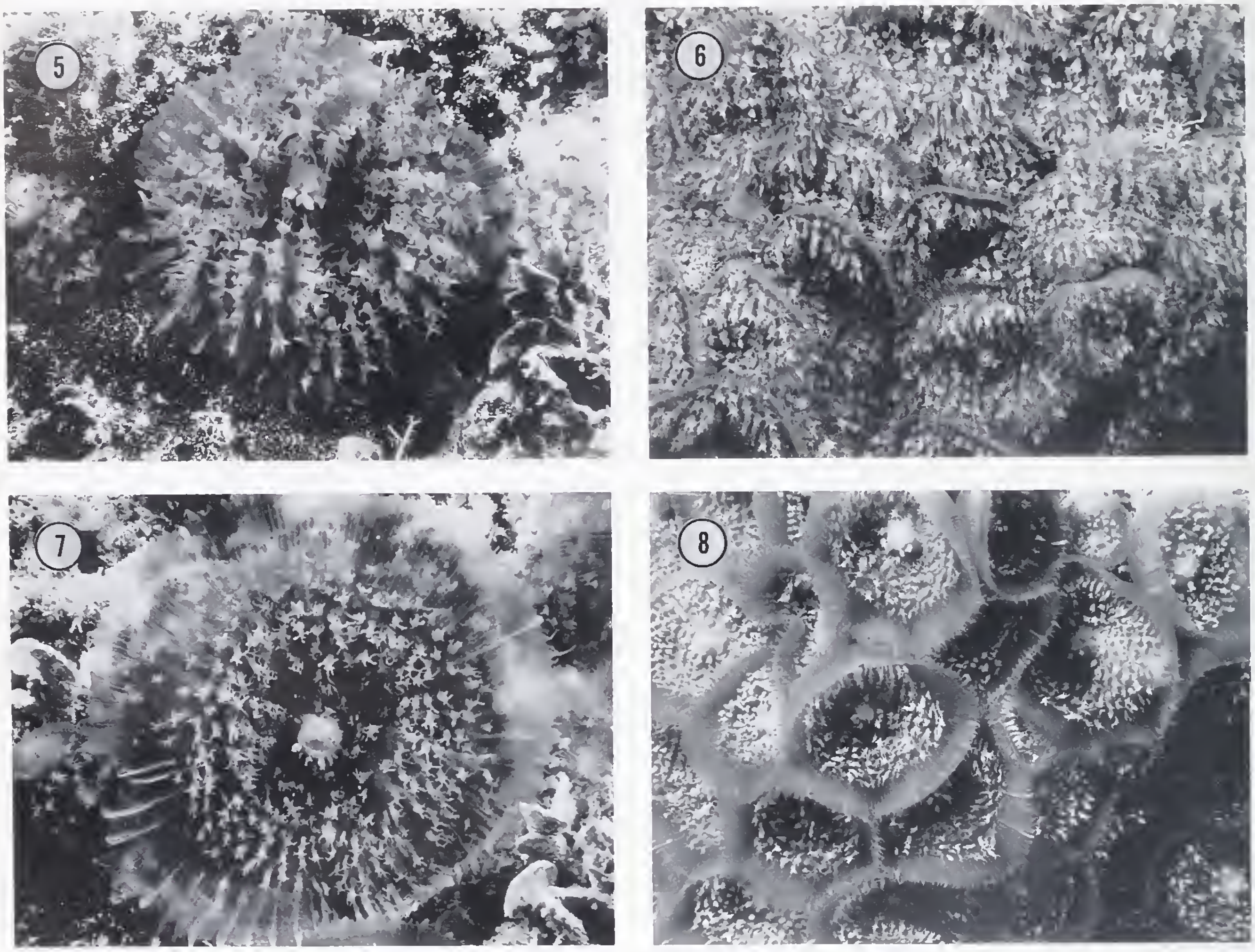


Figures 1–4. Prey capture sequence of *Discosoma sanctithomae*. (1) Feeding posture at night. Margin of the oral disc expanded and turned up to form a bowl-shaped structure. (2) After stimulation the margin of the oral disc contracts forming a cavity with a restricted opening above the mouth that is fringed by the marginal tentacles. (3) Complete closure of the opening above the mouth. (4) Photograph of a different specimen of *D. sanctithomae* that has captured a small fish. Refer to text for a more detailed description of the behavior.

a reasonable estimate of the numbers of prey captured during dawn but underestimates the number of prey captured throughout the night.

No *D. sanctithomae* were observed feeding in the field during the day. However, field specimens did feed on *Artemia* nauplii presented to them from a medicine dropper. The feeding response was not as fast or as effective compared to the same trials done during the night. This was related to the animals change in posture between day and night. During the day the margin of the oral disc was turned down in a convex posture (Figs. 5, 6), but at night the margin of the oral disc expanded and was turned up forming a bowl-shaped structure (Figs. 7, 8). This posture facilitated the capture of prey. When *Artemia* were presented to the oral disc of *D. sanctithomae* during the day, most individuals did not perform an effective envelopment response and few prey were captured. This was because the margin of the oral disc was not expanded and turned upwards allowing the engulfment of a large volume of water when the margin contracted. Instead, when the oral disc margin contracted it was pulled in across the surface of the discal tentacles, or in some individuals the turned-down oral disc margin contracted around the column.

The surface area of the oral disc was significantly smaller during the day than during the night (Figs. 5, 7). Individuals that were separated by open space during the day often overlapped neighboring individuals at night with their expanded oral disc (Figs. 6, 8). Digitized measurements of oral disc surface area (in the plane of the



Figures 5–8. Diel change in posture and pattern of contraction and expansion of the oral disc and tentacles of *Discosoma sanctithomae*. (5) Day-time posture of a single individual with contracted oral disc margin that is turned down toward the substratum and the discal tentacles expanded. (6) Colony of *D. sanctithomae* during the day. (7) Posture at night of the same individual as in Figure 5. Oral disc margin is expanded and is turned up to form a bowl-shaped structure. The discal tentacles are contracted. (8) Same colony of *D. sanctithomae* as in Figure 6 at night.

photograph) taken during the day and night demonstrated no diel difference in the surface area covered by the discal tentacles ($P > 0.05$; Paired *t*-test), but the disc margin expanded to cover a much wider surface area at night (Fig. 9: $P < 0.001$). The discal tentacles were also expanded during the day and contracted at night (Figs. 5–8). This was correlated with the densities of zooxanthellae in the tissues of these different body regions. There were high densities of zooxanthellae in the discal tentacle region (54.5 ± 8.9 cells·mm^{-3}; n = 6), but low densities in the disc margin (15.5 ± 1.4 cells·mm^{-3}; n = 6). This pattern was correlated with the amount of pigmentation in these two regions. The central portion of the oral disc and the discal tentacles were dark green and brown and the margin was relatively transparent when expanded at night (Figs. 5–8).

Animals kept in the laboratory under a varying light/dark cycle of both ambient and fluorescent lighting did not display a distinct diel difference in expansion and contraction or posture. There was a variety of responses by different individuals, with some remaining in an expanded state throughout the day and night and other specimens varying their shape over time.

Both mechanical and chemical stimuli were effective in eliciting the envelopment response. There was a significant diel difference in the number of contacts to the discal tentacles required to elicit envelopment (Fig. 10; $P < 0.001$, Mann-Whitney). In field trials done during

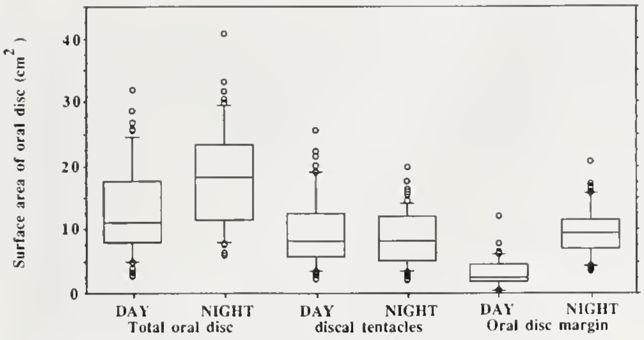


Figure 9. Boxplots for the surface areas of different portions of the oral disc during the day and night. There are significant differences in surface area between day and night for the total oral disc ($P < 0.001$) and the oral disc margin ($P < 0.001$), but not for the discal tentacle region ($P > 0.05$; Paired *t*-tests for each comparison). The central box part of the display (the hinges) indicates the interquartile range, the absolute difference between the 25th and 75th percentiles. The central horizontal line is the median, and the "whiskers" that extend out from the central box indicate the main body of the data and are calculated using the formula: hinge ± 1.5 (interquartile range). Any data values that extend out beyond these limits are plotted with a circle (n = 61).

the day, more than two contacts to the discal tentacles with a pasteur pipette were usually required to elicit the response, but some individuals did not close after even 20 contacts or forceful blows. At night most individuals closed after the first few contacts and exhibited a typical envelopment response. The sensitivity of the animals appeared to be related to their degree of expansion of the oral disc and their posture. In the laboratory, those individuals displaying a typical feeding posture were most sensitive to mechanical stimuli, while animals that were in a normal daytime posture rarely responded, independent of the ambient light levels.

A forceful stream of sea water (approximately 1 ml · s^{-1}) directed at the oral disc from a pipette or syringe was also capable of eliciting the envelopment response. However, gentle streams of seawater (approximately 0.1 ml · s^{-1}) that only lightly swayed the discal tentacles did not elicit envelopment. This allowed the presentation of chemicals in the stream of seawater. In field tests *Artemia* homogenate was very effective at eliciting the envelopment behavior (Table I). Of the 20 amino acids tested, 15 did not cause a response: glycine, serine, threonine, cystine, arginine, tyrosine, lysine, histidine, aspartic acid, valine, leucine, isoleucine, methionine, phenylalanine, and tryptophan. Five amino acids were effective at high concentrations (10^{-1} *M*). However, only proline elicited a consistent response at 10^{-2} *M* and a less strong response at 10^{-3} *M*. The strongest reaction was caused by the tripeptide reduced glutathione (GSH) that was effective to concentrations of 10^{-4} *M*. Both proline and GSH also caused mouth opening, distension of the actinopharynx, and extrusion of mesenterial filaments through the mouth or body wall of the oral disc. We point out that these are the concentrations in the stream of water directed at the animals so that the actual effective concentrations are much lower.

Two chemical analogs of proline, pipecolic acid, and L-hydroxyproline were also effective in eliciting the closure response and subsequent mouth opening behavior at the same concentrations (Table II). Azetidine carboxylic acid was not effective. The glutathione analog, S-methyl glutathione, was effective at approximately the same concentrations as GSH and elicited both closure of the oral disc and mouth opening.

Previous tests demonstrated that mechanical and chemical stimuli elicit oral disc closure. The control of subsequent feeding behaviors was investigated in the laboratory by presenting one or both of these stimuli. Animals that closed in response to being contacted with a pipette and sea water control reopened and resumed their typical posture after two to three minutes. This same behavior was observed for field specimens that closed in response to being contacted by a prey item but were not successful in capturing it. When a solution of proline or GSH was released into the cavity instead of plain seawater, the animal responded by contracting the margin fully, opening its mouth, and distending the actinopharynx as in a typical feeding reaction. The amount of time the animals remained closed before resuming their open posture was significantly greater with GSH or proline than with the seawater control (Fig. 11; $P < 0.05$, Mann-Whitney). The stronger the chemical stimulus the stronger the contractions of the oral disc. Thus it took a longer time for the animal to re-expand and resume a normal feeding posture. In both field and laboratory tests done with GSH (10^{-2} *M*) all animals extruded many

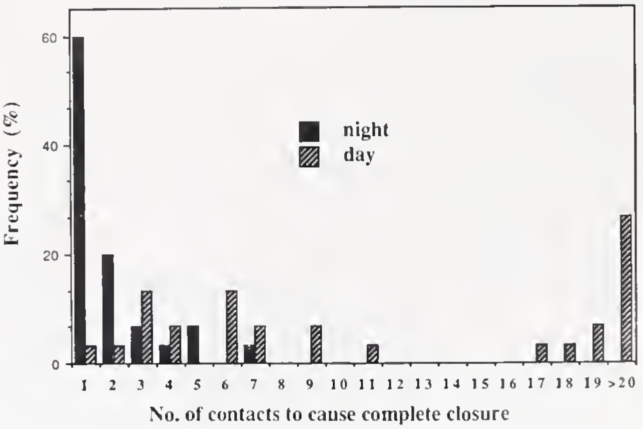


Figure 10. Frequency histograms for the number of contacts to the discal tentacles with a glass pipette that are required to cause complete closure of the oral disc. There was a significant difference in the number of contacts required between the night and day ($P < 0.001$; Mann-Whitney, n = 60).

Table I

Field tests with Artemia *extract and chemicals at various concentrations*

Solution	Concentration (*M*)	Number of trials	# of full closures	# of partial closures	% closure responses
Artemia homogenate		18	18	0	100
Asparagine	10^{-1}	15	15	0	100
	10^{-2}	12	5	2	58
	10^{-3}	8	1	2	38
	10^{-4}	10	0	0	0
Glutamine	10^{-1}	12	12	0	100
	10^{-2}	10	0	0	0
Glutamic acid	10^{-1}	10	10	0	100
	10^{-2}	10	1	2	30
	10^{-3}	8	2	0	25
	10^{-4}	12	0	0	0
Alanine	10^{-1}	10	10	0	100
	10^{-2}	10	1	5	60
	10^{-3}	10	0	0	0
Proline	10^{-1}	15	15*	0	100
	10^{-2}	20	20	0	100
	10^{-3}	10	7	2	90
	10^{-4}	10	0	2	20
	10^{-5}	10	0	0	0
GSH	10^{-1}	18	18*	0	100
	10^{-2}	10	10*	0	100
	10^{-3}	11	11	0	100
	10^{-4}	7	2	2	57
	10^{-5}	12	0	0	0

* Extrusion of mesenterial filaments in some individuals.

Responses of *Discosoma sanctithomae* to a gentle stream of test solution (0.5 ml) from a 5-ml syringe directed at the discal tentacles from a distance of approximately 3 mm. Full closure refers to a complete envelopment response in which the margin of the oral disc was fully contracted closing the aperture to the cavity above the mouth. Partial closures were only slight contractions of the oral disc margin. Other amino acids tested that did not cause a response were glycine, serine, threonine, cystine, arginine, tyrosine, lysine, histidine, aspartic acid, valine, leucine, isoleucine, methionine, phenylalanine, and tryptophan.

Table II

Tests with analogs of proline and glutathione (GSH) in the laboratory

Solution	Concentration (*M*)	Number of trials	# of full closures	# of partial closures	% closure responses
L-azetidine carboxylic acid	10^{-2}	10	0	0	0
Pipecolic acid	10^{-2}	10	10	0	100
	10^{-3}	10	9	0	90
	10^{-4}	10	0	0	0
L-hydroxyproline	10^{-2}	20	20	0	100
	10^{-3}	10	8	1	90
	10^{-4}	10	0	0	0
	10^{-5}	10	0	0	0
S-methyl GSH	10^{-2}	10	10	0	100
	10^{-3}	10	6	3	90
	10^{-4}	10	0	0	0

Responses of *Discosoma sanctithomae* to a stream of filtered sea water (0.5 ml; with and without dissolved chemicals) at various concentration from a glass pipette directed at the discal tentacles from a distance of approximately 3 mm.

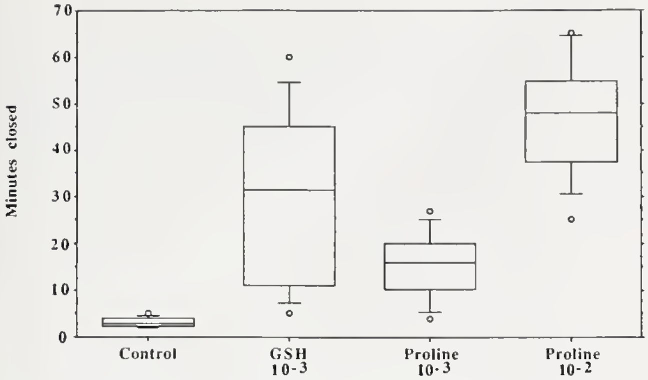


Figure 11. Boxplots for the number of minutes the oral disc of *Discosoma sanctithomae* remained closed after being stimulated with a light touch of a glass pipette and then 0.5 ml of solution being injected into the cavity formed by the margin of the oral disc. The control was plain seawater and the treatments were solutions of glutathione and proline. Ten trials were performed for the control and each treatment in laboratory tests. Each treatment was significantly different from the control ($P < 0.001$; Mann-Whitney tests), and proline (10^{-2} *M*) was significantly different from the other two treatments ($P < 0.05$; Mann-Whitney tests). Proline (10^{-3} *M*) was not significantly different from GSH (10^{-3} *M*) ($P > 0.05$; Mann-Whitney test).

mesenterial filaments and did not reopen for several hours. The time specimens remained closed was greater for animals stimulated with proline (10^{-2} *M*) than for proline (10^{-3} *M*) and GSH (10^{-3} *M*) ($P < 0.05$, Mann-Whitney tests). There was no significant difference between GSH (10^{-3} *M*) and proline (10^{-3} *M*) ($P > 0.05$, Mann-Whitney test).

Tests were done with filter papers (with and without chemicals) to determine which chemicals elicited the envelopment response and subsequent ingestion (Table III). Most animals did not respond to plain pieces of filter paper that were dropped onto the oral disc (>93% of trials), and the papers were rarely ingested (<2% of trials). All trials were done by first dropping a plain piece of filter paper onto the oral disc and if there was no response then a treated piece of paper was tested. Chemicals effective only at high concentrations in the field syringe tests did not elicit consistent closure or ingestion in these tests. Proline and GSH-treated filter papers caused immediate closure of the oral disc, but GSH was effective at lower concentrations. Only GSH elicited consistent ingestion. Some filter papers with GSH that were not ingested were covered with mesenterial filaments extruded through the mouth or body wall of the oral disc. Similar responses occurred in tests using plain pieces of filter paper and then eliciting closure with 0.5 ml of GSH or proline from a pipette.

Pipecolic acid treated filter papers caused closure and were ingested at a concentration of 10^{-1} *M* but were not effective at 10^{-2} *M* (Table IV). Hydroxyproline and azetidine carboxylic acid treated papers did not elicit any consistent response. The responses to S-methyl GSH were much similar to GSH and resulted in closure, ingestion, and extrusion of mesenterial filaments.

Discussion

Many cnidarians use the products of their endosymbiotic zooxanthellae as a major source of nutrients. It has been suggested that the Discosomatidae of the Caribbean may be primarily, if not exclusively, dependent on these associates for nutrition (Den Hartog, 1980). However, cnidarians also require exogenous sources of nitrogen, phosphorus, essential amino acids, and trace elements not provided by their symbionts. These are usually obtained through feeding on benthic or planktonic prey (Muscatine and Porter, 1977; Van-Praët, 1985; Sebens, 1987). The disc-shaped morphology of the Discosomatidae is probably adaptive for the collection of solar radiation (Den Hartog, 1980), but their flattened shape is also effective in allowing the capture of prey by envelopment.

Indo-Pacific Discosomatidae (= Actinodiscidae) have a similar morphology and utilize the same type of prey capture technique as *D. sanctithomae* (Hamner and Dunn, 1980). The largest Indo-Pacific species, *Amplexidiscus fenestrafer*, can envelop prey almost as quickly as *D. sanctithomae* (within 3 s after contact). Two smaller species (*Actinodiscus fungiformis* and *Rhodactis howesii*) close much more slowly (10 s–1 min). Colonies of these three species are found in well-lit reef areas on vertical walls, much like populations of *D. sanctithomae* in Bermuda. This orientation may facilitate the capture of large planktonic organisms that are swept into contact with the oral disc. An engulfment feeding method may be more effective in the capture of large, active prey than tentacular adhesion.

Our observations of *D. sanctithomae* capturing polychaetes and fish that were attracted to dive lights during night dives suggests that these corallimorpharians are capable of capturing large mobile prey. However, the few prey that were found in the coelenterons of specimens collected from the field at dawn were not of this type. The small number of prey found in our samples is comparable to some of the analyses of coral coelenterons examined by Porter (1974), but on the average he found a higher proportion of coral polyps feeding and containing multiple prey items. The relative lack of food items encountered in our analysis may be related to the small sample size (63 specimens), the small numbers of prey in the plankton during May, or be an artifact of the morning collection time. Further coelenteron analyses from animals collected at different times of the year and night are required to better establish the natural diet of *D. sanctithomae*.

Table III

Responses of Discosoma sanctithomae *to filter papers (saturated with solutions with and without chemicals) dropped onto the oral disc*

Filter paper	Concentration (M)	Number of trials	# of full closures	# ingestion responses	% ingestion responses
Control (no chemicals)		185	12	2	1
Asparagine	10^{-1}	5	0	0	0
Glutamine	10^{-1}	5	0	0	0
Glutamic acid	10^{-1}	5	0	0	0
Alanine	10^{-1}	6	3	3	50
	10^{-2}	5	0	0	0
Proline	10^{-1}	5	5	0	0
	10^{-2}	10	3	1*	10
	10^{-3}	5	0	0	0
GSH	10^{-1}	5	5	4*	80
	10^{-2}	22	22	16*	73
	10^{-3}	10	10	7*	70
	10^{-4}	10	8	1	10
	10^{-5}	10	0	0	0

* Extrusion of mesenterial filaments in some specimens.

Many coral reef anthozoans have diel patterns of expansion and contraction that are related to both light and prey availability (Porter, 1974; Gladfelter, 1975; Sebens and DeReimer, 1977). Structures that have high densities of zooxanthellae are expanded during the day and are retracted at night. Those with few or no zooxanthellae are retracted during the day and are expanded at night when demersal zooplankton are most abundant. Sebens and DeReimer (1977) did not report that *Discosoma* (= *Rhodactis*) *sanctithomae* had a change in posture or a diel pattern of expansion and contraction of the margin of the oral disc and discal tentacles. However, the pattern observed in our study supports their general observations. The oral disc and discal tentacles have high densities of zooxanthellae and are expanded during the day. The margin of the oral disc, which has relatively few zooxanthellae, is greatly expanded at night compared to during the day. Expansion at night is adaptive for the capture of prey and contraction during the day may be a strategy to reduce metabolic costs or shading of clonemate neighbors when the probability of capturing prey is low (Sebens and DeReimer, 1977).

These differences in expansion and contraction may not be as conspicuous as in other species of cnidarians that have been studied (Sebens and DeReimer, 1977), but a slight change in the posture of *D. sanctithomae* had

Table IV

Responses of Discosoma sanctithomae *to filter papers saturated with solutions of proline and glutathione analogs*

Filter paper	Concentration (M)	Number of trials	# of full closures	# ingestion responses	% ingestion responses
L-azetidine carboxylic acid	10^{-2}	6	0	0	0
Pipecolic acid	10^{-1}	10	9	8	80
	10^{-2}	5	1	0	0
L-hydroxyproline	10^{-1}	5	1	1	20
	10^{-2}	5	0	0	0
S-methyl GSH	10^{-2}	10	10	7*	70
	10^{-3}	9	7	1	11
	10^{-4}	10	6	0	0
	10^{-5}	10	0	0	0

* Extrusion of mesenterial filaments in some specimens.

a major effect on its ability to capture prey. Individuals not in a bowl-shaped posture were unable to exhibit an effective envelopment response or did not respond at all. Den Hartog (1980) suggests that the ability of *D. sanctithomae* to vary the shape of the oral disc from concave to convex may be an adaptation to receive an optimal amount of solar radiation during the day, but this change in shape is probably more related to maximizing the capture of prey that are more abundant in the water column at night. The difference in sensitivity to physical contact between day and night appeared to be related to the posture of the animals. Animals kept in the laboratory under a varying light regime did not exhibit a diel pattern of expansion and contraction. Independent of the time of day or light intensity, only those individuals that were in an expanded feeding posture displayed a consistent envelopment response. There may be a certain physiological state associated with the feeding posture that increases the sensitivity of the animals to mechanical stimuli. Other cnidarians are more sensitive toward feeding stimuli when they are in a normal feeding posture (Williams, 1972). Further neurophysiological studies of the diel behavior patterns of corallimorpharians may prove to be interesting.

Both mechanical and chemical stimuli are generally required to elicit a complete feeding response in most cnidarians (Lenhoff and Lindstedt, 1974). Strictly mechanical stimuli do not elicit consistent mouth opening and ingestion (Williams, 1972). Contact with a glass pipette to the oral disc of *D. sanctithomae* caused only closure and specimens reopened after a few minutes to resume a normal feeding posture. Purely mechanical stimuli will also cause envelopment by *A. fenestrafer* (Hamner and Dunn, 1980).

Chemical stimuli elicited both closure and further feeding behaviors in *D. sanctithomae,* but the source of these activators is not readily explained. Most studies of cnidarian feeding behavior assume that mouth opening and ingestion are a response to the release of body fluids (which contain numerous compounds including various feeding activators) from the prey liberated from puncture wounds made by nematocyst discharge during capture. There are few nematocysts in the tentacles of *D. sanctithomae,* and no nematocysts were discharged onto items that were put in contact with the discal tentacles. Prey did not appear to be stung by these tentacles after capture but they may be stung by extruded mesenterial filaments, nematocysts on the actinopharynx, or the marginal tentacles if they try to escape through the constricted opening of the cavity formed by the margins of the oral disc. Nematocysts were discharged onto objects presented to the marginal tentacles and they could be involved in releasing feeding activators from the prey. External digestion by mesenterial filaments extruded out of the mouth or other regions of the oral disc has been described for *Rhodactis howesii* (Hamner and Dunn, 1980) and was observed for treated filter papers in *D. sanctithomae.* These filaments have high densities of holotrichous nematocyts (Den Hartog, 1980) that could puncture prey and liberate body fluids. There may only need to be a few nematocysts discharged to puncture the prey enough to create a point source of chemical to elicit feeding. Reimer (1971) suggests that the concentrations required for many feeding activators to elicit a response (10^{-4} *M* and higher) are too high to be ecologically significant since they are not likely to occur under natural conditions. However, if these chemicals are being released into a confined space, such as the cavity formed by the contraction of the oral disc in the Discosomatidae, then high concentrations may occur. Other sources of feeding activators may be components of prey excretory products or the epidermal covering of the prey (*e.g.,* fish mucus). Lubbock (1980) has shown that the mucus of some fish species can elicit prey capture and ingestion by sea anemones.

The concentrations of chemicals tested in our experiments were similar to those used in other studies. In filter paper tests both corals (Lehman and Porter, 1973) and sea anemones (Reimer, 1973) respond to filter papers soaked in solutions of chemicals as dilute as 10^{-3} *M,* much the same as the responses of *D. sanctithomae* to both proline and GSH. In tests where whole animals are placed in solutions, responses have been recorded to concentrations as low as 10^{-7} *M* (Mariscal and Lenhoff, 1968). We did not perform this type of experiment, but the effective concentrations of both GSH and proline may be comparable in our experiments in which solutions were released from syringes in the general direction of the corallimorpharians. The concentrations of the chemicals would have been much lower once they reached the animals because of rapid dilution in the surrounding seawater.

The imino acid proline and the tripeptide GSH are widespread feeding activators in cnidarians (reviews by Lenhoff and Lindstedt, 1974; Lenhoff *et al.,* 1976; Van-Praët, 1985; Sebens, 1987). These activators, along with various other amino acids and chemicals, cause both preparatory feeding and ingestion responses in different groups of anthozoans. In some species different activators cause different parts of the feeding response (Lindstedt, 1971; Reimer, 1971, 1973; Williams, 1972; Nagai and Nagai, 1973; Bursey and Guanciale, 1977). This was also observed for *D. sanctithomae.* Proline, GSH, and some other amino acids elicited envelopment at high concentrations, but only GSH caused a complete feeding sequence ending in ingestion. Proline and GSH have been reported to cause ingestion behaviors in both corals and sea anemones (Lenhoff and Lindstedt, 1974; Van-

Praët, 1985). Mesenterial filaments were extruded only at high concentrations of proline (10^{-1} *M*), whereas GSH elicited mesenterial filament extrusion at concentrations as low as 10^{-3} *M*. Similar differences in response to these chemicals have been reported for corals (Mariscal and Lenhoff, 1968). The differences in behavior elicited by proline and GSH, along with the knowledge that proline has a distinctly different chemical structure from GSH, suggests that these activators have different receptors and different connections to the neuronal network involved in the feeding response.

Trials with proline and GSH analogs revealed some characteristics of the receptor(s) for these chemicals. Two proline analogs, pipecolic acid and L-hydroxyproline, were as effective at causing envelopment as the naturally occurring compound. The proline analog L-azetidine carboxylic acid did not elicit any behavior. This suggests that the size of the ring structure is important, but additional side chains do not alter the recognition of this group of compounds. Reimer (1971) reported a different result for the zoanthid *Palythoa*, in which changes in the size of the ring structure did not alter its activity. Also, Reimer (1973) found that addition of an OH-group (hydroxyproline) caused a response in the sea anemone *Calliactis polypus*. The analog S-methyl glutathione also reacted in the same fashion as GSH. To better establish the structural requirements of the receptor(s) involved in these feeding behaviors further tests should be conducted for inhibition and competition with particular chemical analogs.

Acknowledgments

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Behavioral Responses of Crustacean Larvae to Rates of Salinity Change

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Abstract. The ontogeny of behavioral responses of larvae of the crabs *Rhithropanopeus harrisii* and *Neopanope sayi* to rates of change in salinity were analyzed with a video system. A salinity increase evoked an ascent in both species. For *R. harrisii* the threshold rate of increase was 1.1×10^{-3} ppt s^{-1} for the first and last zoeal stages and changed little with acclimation salinity. *N. sayi* larvae were more sensitive, as thresholds were 2.8×10^{-4} ppt s^{-1} for Stage I zoeae and 7.0×10^{-4} ppt s^{-1} for Stage IV. This difference in sensitivity may relate to the magnitude of salinity gradients in the estuarine/coastal areas inhabited by the larvae. At threshold rates of salinity increase the absolute amount of change before a response was lower for Stage I zoeae (0.09–0.11 ppt) than Stage IV zoeae (0.21–0.29 ppt) for both species. Decreases in salinity did not induce the expected descent response in either species at rates up to 4.7×10^{-2} ppt s^{-1}. The different responses in a salinity gradient may have resulted because the rate threshold and absolute amount of change before a response to a salinity increase were below those for a salinity decrease. Considering larval sinking rates and normal environmental salinity gradients, larvae of both species can respond to rates and amounts of salinity increase in their environment. The ascent response may be important for keeping larvae up in the water column and reducing the likelihood that they will encounter the bottom.

Introduction

A change in salinity dramatically modifies the behavior of many species of crustacean larva (Sulkin, 1984; Forward, 1987). Considering those cases where responses are reported, there is a characteristic pattern of responsiveness. A decrease in salinity, as occurs high in a stratified water column, induces downward movement (Lance, 1962; Scarratt and Raine, 1967; Harder, 1968; Hughes, 1969; Roberts, 1971). The underlying responses include positive geotaxis (Latz and Forward, 1977; Sulkin *et al.*, 1980), passive sinking (Latz and Forward, 1977), and negative phototaxis (Lyon, 1906; Edmondson and Ingram, 1939; Latz and Forward, 1977). Increases in salinity usually occur low in the water column and generally evoke upward movement. This ascent can result from negative geotaxis (Latz and Forward, 1977; Sulkin *et al.*, 1980), an increase in swimming speed (Sulkin *et al.*, 1980), and positive phototaxis (Latz and Forward, 1977; Sulkin and Van Heukelem, 1982).

Thus past studies indicate that larvae have a negative feedback system in which salinity conditions high in the water column induce downward movement and those low in the water column induce the opposite responses. This system could function for depth maintenance according to salinity. However, for this to occur, larvae must have sufficient sensitivity to detect the actual salinity changes that occur in their environment.

The minimum step change in salinity (threshold) necessary to induce behavioral responses varies with species. The threshold for a reversal in phototactic sign for larvae of the crab *Rhithropanopeus harrisii* upon step decreases in salinity ranges from 1.1 to 2.0 ppt (Latz and Forward, 1977). Measurements in haloclines indicate that some zoeal stages of *Callinectes sapidus* (Sulkin and Van Heukelem, 1982) and *Pagurus longicarpus* (Roberts, 1971) can detect salinity discontinuities at least as low as 2.5 ppt, while *Uca pugnax* requires a change of 6 ppt (O'Connor and Epifanio, 1985).

The problem with these past studies is that larvae were exposed to step changes in salinity or sharp haloclines.

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In the field larvae encounter vertical gradients in salinity and less commonly experience sharp haloclines. Thus in the water column larvae perceive changes in salinity that depend upon the magnitude of the vertical gradient and rates of vertical movement.

The present study was undertaken (1) to determine the lowest rates of salinity change that evoke behavioral responses, (2) to compare these rates to those that a larva could encounter in the water column, and (3) to determine the absolute amount of salinity change that must occur before a response. The study compared larvae of the crabs *Rhithropanopeus harrisii* and *Neopanope sayi*. These species were used because both live as adults in estuaries but the behavior of *R. harrisii* larvae results in retention in upper estuarine areas (Cronin, 1982), while *N. sayi* larvae are not retained and undergo development in lower estuarine and coastal areas (Sandifer, 1975; Dittel and Epifanio, 1982; Salmon *et al.*, 1986). Thus each larval species is potentially exposed to a different salinity regime, since salinity gradients are greater in estuarine than coastal areas.

Materials and Methods

Ovigerous *Rhithropanopeus harrisii* were collected from the Neuse River estuary (North Carolina) from May to August, 1988. Crabs were placed in either 10 or 20 ppt seawater (acclimation salinity), which was passed through a 5-μ filter. Since the females adjust their body fluid osmolality to salinity changes in 8 h (Diamond *et al.*, 1989), it was assumed that the embryos were similarly adjusted at the time of hatching, which occurred after 8 h in the acclimation salinities. Groups of larvae were reared in both salinities and experiments conducted with Stages I and IV zoeae. Even though the upper salinity (20 ppt) is optimal for developmental success, larvae can complete development in 10 ppt (Costlow *et al.*, 1966).

Ovigerous *Neopanope sayi* (Smith) were collected near the entrance to the Newport River estuary (North Carolina) from June to September, 1988, and females placed in 28 ppt seawater (acclimation salinity). Larvae were reared at this salinity and Stages I and IV zoeae used in the experiments. This salinity was selected because it is close to the salinities where maximum larval abundance occurs in the field (Sandifer, 1975) and to permit the experimental salinity changes to encompass normal salinities encountered by the larvae. Responses after acclimation to a lower salinity were not tested because there was almost no change in sensitivity by *R. harrisii* larvae when acclimated to different salinities. Stages I and IV zoeae were tested for an ontogenetic change in responsiveness because each species has four zoeal stages.

Rearing took place in a controlled environmental chamber (Sherer, Model CEL4-4) on a 14:10 LD cycle and 25°C. Throughout development, larvae were transferred daily to clean seawater and fed newly hatched *Artemia* spp. nauplii. All experiments were performed in the 6-h interval in the middle of the light phase to avoid complications due to biological rhythms in behavior. Larvae were light-adapted to room fluorescent light (intensity = about 1 W m^{-2}) prior to all experiments. In most cases a minimum of five groups of larvae, each from a separate female, were tested in each experiment.

Experimental approach

Larval responses to different rates of salinity change were measured in a chamber having three vertical cylindrical sections (section height = 2.5 cm; diameter = 2.5 cm; Fig. 1). For a salinity decrease, low salinity water was added to the upper section and mixed by a slowly rotating stirring paddle. Larvae were confined to the middle section by plankton netting (75 μ mesh) at the upper and lower boundaries, and their behavior monitored and recorded with a closed circuit television system. For viewing, animals were illuminated with far-red light (maximum transmission 775 nm), to which larvae are not responsive (Forward and Cronin, 1979). The lower section was used for salinity increases. High salinity water was added and mixed with a magnetic stirring bar. Preliminary measurements of larval swimming indicated slow stirring in the upper and lower sections had no apparent effect on movement.

To insure that there were constant amounts of water in all sections and constant flow rates through the center section, the different salinities were delivered to the appropriate end section by a variable speed peristaltic pump, and water was extracted at the same rate from the other end section by the same pump. For example, to induce a salinity decrease, low salinity water was pumped into the upper chamber, while water was removed from the lower chamber at the same rate. Dye studies indicated the flow of water through the netting into the center section was approximately laminar.

The rates of change in salinity were varied through differences between input and acclimation salinity and pumping rates. The actual salinity in the center of the larval section was measured with a conductivity probe (Model PP1042; Radiometer), which was calibrated in the center section with water of known salinity. The smallest detectable change in salinity was about 0.09 ppt. Water temperature remained relatively constant at 25°C during calibration and throughout the experiments. The digital readout of conductivity was viewed by a second video camera and inserted in the video picture with a video screen splitter (Model U2705P; Vicon Industries, Inc.). A record of time was also inserted in the picture by

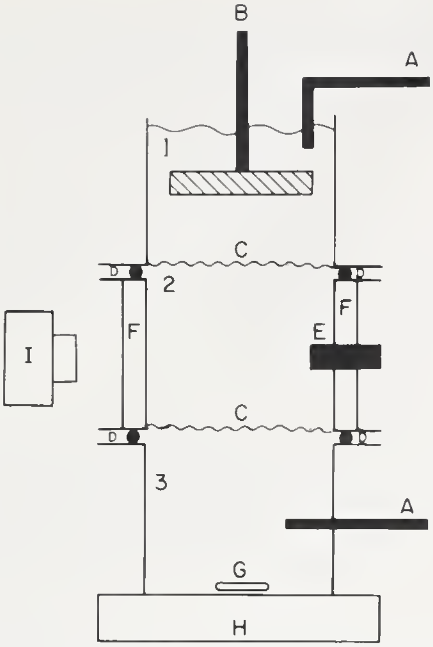

Figure 1. Horizontal view of the test chamber consisting of equal size cylindrical upper (1), test (2), and lower (3) sections (not drawn to scale). A—fluid input/output tube connected to the peristaltic pump; B—stirring paddle connected to a variable speed stirring motor; C—75 μ mesh plankton netting; D—O-ring; E—conductivity probe connected to meter with digital readout; F—rectangular water-filled chamber surrounding the test chamber; G—magnetic stirring bar; H—magnetic stirrer; I—video camera; J—far-red illumination light. In the actual chamber the video camera and conductivity probe are oriented perpendicular to each other.

a Field/Frame Counter (QSI Systems, Inc.). In this way larval behavior, conductivity, and time were recorded simultaneously on video tape. The actual rates of change in salinity were calculated from the measurements of time and conductivity. In each experiment the rate of change in salinity quickly increased up to a maximal rate for each flow rate/salinity difference condition and then remained approximately uniform through the time when responses were measured.

Experimental procedures

The same general procedure tested for responses to salinity increases and decreases. A group of approximately 75 Stage I or 25 Stage IV zoeae was placed in the test chamber and after 1 min in darkness the peristaltic pump and videotape recorded were started. The experiment continued until at least 1 ppt change occurred upon a salinity increase and 5 ppt change upon a decrease. Any response was evident after this amount of salinity change. Larvae were then removed, the chamber rinsed with acclimation salinity water, and a new group of larvae placed in the chamber. Larvae were only tested once at each developmental stage. To control for responses to flow through the chamber, larvae were tested using the above procedure at the maximum test flow rate with acclimation salinity water.

Analysis

All experiments were conducted with the test chamber in a light-tight space. Thus larvae were only illuminated with far-red light. Since they were functionally in darkness in this situation, the possible behavioral responses to changes in salinity were changes in activity and/or geotaxis.

To analyze for behavioral responses, the larval section of the test chamber was divided into three equal horizontal subsections by a template placed over the video screen. The number of larvae in each subsection was counted before (control) and after a change in salinity, and the percentage of larvae in each subsection was calculated. When testing different rates of salinity change, responses were measured after a 0.75 ppt change for a salinity increase and a 5.0 ppt change for a salinity decrease. These absolute changes were used because responses were clearly evident (Fig. 6) for a salinity increase of 0.75 ppt and a 5.0 ppt decrease is about 3-fold larger than the threshold for step decreases for *R. harrisii* larvae (Latz and Forward, 1977). Thus responses to salinity decreases should have been evident by this amount of change.

Ascent and descent responses were expected upon a salinity increase and decrease, respectively. Thus the change in the percentage of larvae in the upper section was monitored upon a salinity increase and in the lower section upon a salinity decrease. Arcsine transformed data were used for statistical tests and to calculate means and standard errors. Back transformed means and standard errors are plotted in the figures. If paired observations were made before (control) and upon stimulation (experimental) of each group of animals, a *t*-test for paired comparisons was used to test for differences. In cases where a control was compared to responses at different times after the beginning of stimulation, then the Dunnett's *t*-test for multiple comparisons with a control was used to test for significant differences ($P < 0.05$; Dunnett 1964).

Results

Salinity increase

Larvae were extremely sensitive to a salinity increase (Fig. 2A). These responses were not due to fluid flow through the chamber, as significant changes in larvae dis-

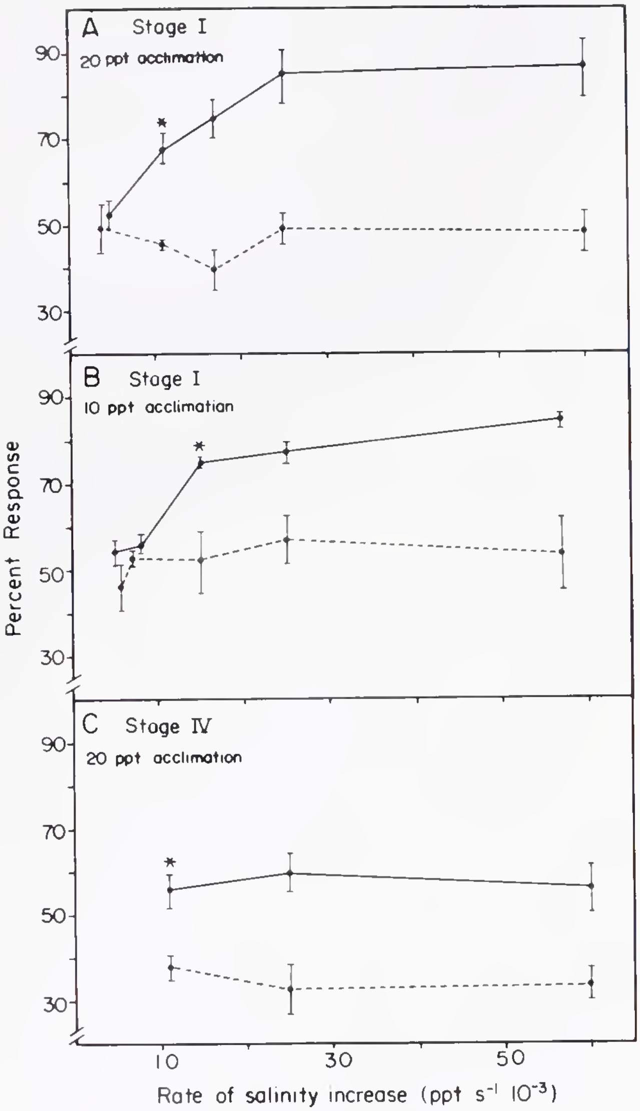

Figure 2. The percentage of *Rhithropanopeus harrisii* larvae aggregating in the upper section of the test chamber before stimulation (control—dashed line) and after 0.75 ppt salinity increase (experimental—solid line) for Stage I zoeae acclimated to 20 ppt (A), and 10 ppt (B), and Stage IV zoeae acclimated to 20 ppt (C). The average number of determinations for each rate was 5 and means and standard errors are plotted. The asterisks indicate the slowest rate (threshold) to induce a response that was significantly ($P < 0.05$) greater than the control.

tributions did not occur under conditions of flow alone (no salinity change) at the fastest test flow rate (Table I).

For *R. harrisii* Stage I zoeae the lowest rates of salinity increase (thresholds) to induce a significant ascent response upon acclimation to 20 ppt (Fig. 2A) and 10 ppt (Fig. 2B) were 1.[illegible] × 10^{-3} and 1.5 × 10^{-3} ppt s^{-1}, respectively. Since these thresholds are similar and in both cases responses were measured after the same absolute amount of salinity change (0.75 ppt), the larvae responded to either the absolute amount or rate of change in salinity and not the absolute levels of salinity. Stage IV zoeae (Fig. 2C) had similar sensitivity, as they displayed a significant response at the lowest test rate (1.1 × 10^{-3} ppt s^{-1}). Since this rate is either at or slightly above the threshold, it will be used as the threshold in future discussions.

N. sayi larvae were more sensitive to a salinity increase than *R. harrisii* larvae. Stage I zoeae of *N. sayi* responded to the lowest test rate (2.8 × 10^{-4} ppt s^{-1}; Fig. 3a), whereas Stage IV were less sensitive with a threshold at 7.0 × 10^{-4} ppt s^{-1} (Fig. 3b).

Salinity decrease

Both species of larvae were unresponsive to salinity decreases. The responses were unaffected by flow from the top to the bottom of the test chamber, as the distributions of larvae in the lower section did not change significantly upon exposure to the fastest test rates of flow alone (Table I).

Responses of *R. harrisii* larvae after a 5-ppt change in salinity are shown in Figure 4. Upon acclimation to 20 ppt, Stage I zoeae did not show a significant descent re-

Table I

Control for flow

Condition	Flow rate (ml/min)	Control m (%)	Control SD	Experimental m (%)	Experimental SD
Salinity increase					
Rhithropanopeus harrisii					
Stage I—20 ppt	7.2	46.0	6.1	39.7	6.7
Stage I—10 ppt	4.1	44.7	5.6	43.7	4.2
Stage IV—20 ppt	4.1	35.2	7.9	33.5	7.0
Neopanope sayi					
Stage I—28 ppt	4.1	41.3	11.2	48.5	4.1
Stage IV—28 ppt	1.94	38.3	10.1	42.8	15.3
Salinity decrease					
R. harrisii					
Stage I—20 ppt	4.1	35.3	4.9	39.4	7.2
Stage IV—20 ppt	7.2	42.3	4.5	52.9	9.9
N. sayi					
Stage I—28 ppt	4.1	38.8	4.3	42.4	2.6
Stage IV—28 ppt	4.1	52.4	6.5	40.6	10.1

The percentage of larvae in the upper section upon a simulated salinity increase and in the lower section with a simulated salinity decrease. Responses are shown before experimentation (control) and after exposure to the maximum experimental flow rate (experimental) of acclimation salinity water through the test chamber. Experimental values were measured at the same time after the beginning of flow as measurements were made upon a real salinity change. The number of groups of larvae tested was 5 in all cases and means (m) and standard deviation (SD) are shown for arcsine transformed data. In no case was there a significant difference between the experimental and control means.

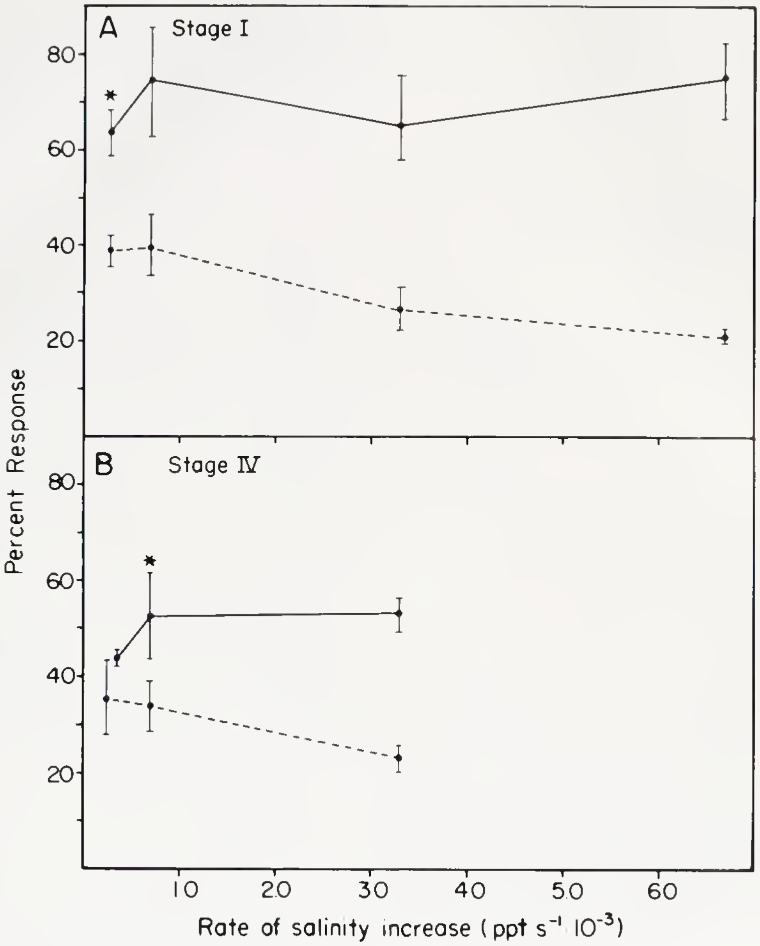

Figure 3. The percentage of *Neopanope sayi* larvae aggregating in the upper section of the test chamber before stimulation (Control—dashed line) and after 0.75 ppt salinity increase (Experimental—solid line) for Stages I (A) and IV (B) zoeae acclimated to 28 ppt. Five determinations were made for each rate, and means and standard errors are plotted. The asterisks indicate the slowest rate (threshold) that induced a significant ($P < 0.05$) response.

sponse (Fig. 4A) at test rates up to the fastest rate possible with available equipment (4.1×10^{-2} ppt s^{-1}). The test rates ranged from those used in the salinity increase experiments (Fig. 2) to an order of magnitude faster. When acclimated to 10 ppt, Stage I zoeae also failed to respond (transformed values, control m = 39.5%, SD = 5.2; experimental m = 42.7%, SD = 1.9; n = 5) at the fastest possible test rate (2.8×10^{-3} ppt s^{-1}). Stage IV zoeae were similarly unresponsive (Fig. 4B).

Responses of *N. sayi* larvae are also shown after a 5-ppt decrease in salinity (Fig. 5). Responses of Stages I and IV zoeae were not significantly greater than control levels. Thus *N. sayi* larvae were unresponsive to salinity decreases up to the fastest test rates (Stage I, 3.5×10^{-2} ppt s^{-1}; Stage IV, 4.7×10^{-2} ppt s^{-1}).

Absolute salinity increase

Responses upon absolute amounts of salinity increase were measured at threshold rates of change. For *R. harrisii*, different amounts of salinity change were required for different zoeal stages, as a significant response occurred after 0.09 ppt change for Stage I zoeae and 0.29 ppt for Stage IV zoeae (Fig. 6A, B). The decrease in sensitivity with later zoeal stages was also observed for *N. sayi* larvae. Stage I zoeae responded after 0.11 ppt change while Stage IV zoeae required 0.21 ppt change (Fig. 6C, D).

Discussion

The expected responses of zooplankton to step salinity changes are an ascent upon a salinity increase and descent upon a salinity decrease (Forward, 1976). In the present experiments with salinity gradients, crab larvae displayed the ascent response but the descent response was absent. Since all experiments were conducted in darkness, the ascent resulted from an increase in swimming speed or negative geotaxis (Forward, 1988).

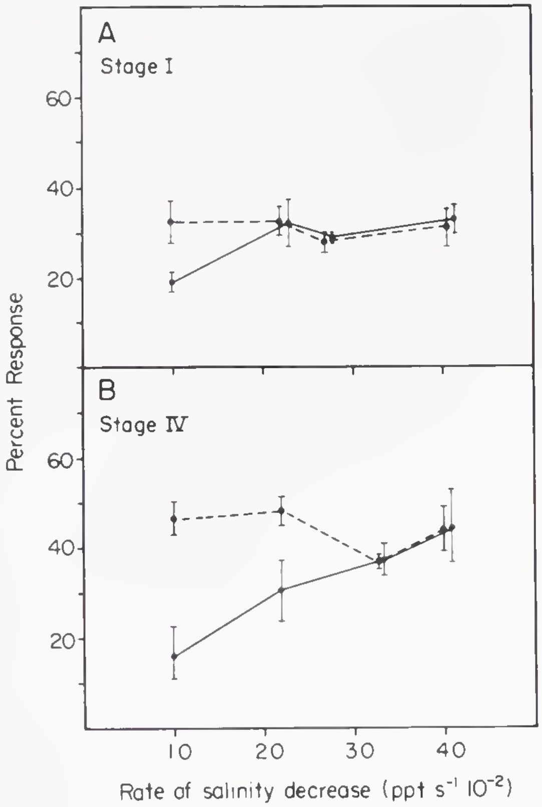

Figure 4. The percentage of *Rhithropanopeus harrisii* larvae aggregating in the lower section of the test chamber before stimulation (Control—dashed line) and after 5.0 ppt salinity change (Experimental—solid line) for Stages I (A) and IV (B). Zoeae were acclimated to 20 ppt. Five determinations were made for each rate, and means and standard errors are plotted.

The test species were selected because *R. harrisii* larvae undergo development in upper estuarine areas (Cronin, 1982), while *N. sayi* larvae develop in lower estuarine and coastal areas (Sandifer, 1975; Dittel and Epifanio, 1982; Salmon *et al.*, 1986). The lowest rate of salinity increase to evoke an ascent response by *R. harrisii* (1.1×10^{-3} ppt s^{-1}) was similar throughout zoeal development and changed little with acclimation salinity. Thus larvae respond to the change in salinity not to an absolute salinity. These results are similar to those found using step changes in salinity (Latz and Forward, 1977). Stages I and IV zoeae of *N. sayi* showed the same behavioral responses but the thresholds (Stage I, 2.8×10^{-4} ppt s^{-1}; Stage IV, 7.0×10^{-4} ppt s^{-1}) were much lower than those for *R. harrisii* larvae. This increased sensitivity by *N. sayi* larvae may result from the environmental salinities zoeae encounter, since larger gradients exist in upper estuarine areas (*e.g.*, Cronin, 1982) than lower estuarine/coastal areas (*e.g.*, Williams *et al.*, 1967).

For some species there are ontogenetic changes in responses to salinity. In the present study Stages I and IV zoeae of both species displayed small differences in sensitivity to a salinity increase. The minimum salinity sensitivity is characterized by the threshold rate of change and the absolute amount of change before a response. The thresholds were the same for both first and last zoeal stages of *R. harrisii*, but for *N. sayi*, Stage I zoeae were 2.5 times more sensitive than Stage IV zoeae. At threshold rates of salinity increase, the absolute amount of change before an ascent response was always less for Stage I than Stage IV zoeae of both species. Thus older larvae appear to be less sensitive to a salinity increase.

None of the test larvae showed descent responses to a salinity decrease in a gradient. The absence of a descent response is unexpected because it has been observed in other species upon step changes in salinity (Lance, 1962; Scarratt and Raine, 1967; Harder, 1968; Hughes, 1969; Roberts, 1971), and a positive geotaxis clearly occurs in Stages I and IV zoeae of *R. harrisii* upon step decreases in salinity (Latz and Forward, 1977). The reason for this discrepancy may be the difference between responses upon step changes and in gradients.

When larvae are tested with a step decrease in salinity, they are exposed to a constant change in salinity that is uniform throughout the test chamber. Thus when larvae

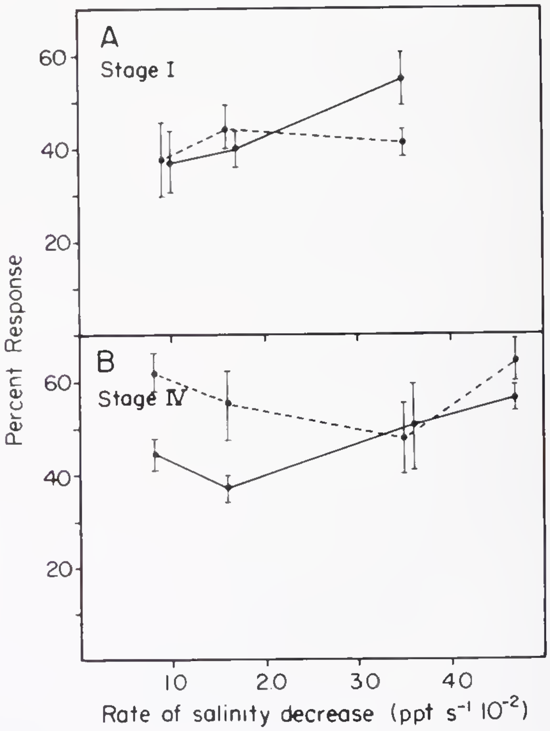

Figure 5. The percentage of *Neopanope sayi* larvae aggregating in the lower section of the test chamber before stimulation (Control—dashed line) and after 5.0 ppt salinity change (Experimental—solid line) for Stages I (A) and IV (B) zoeae acclimated to 28 ppt. Five determinations were made at each rate and means and standard errors are plotted.

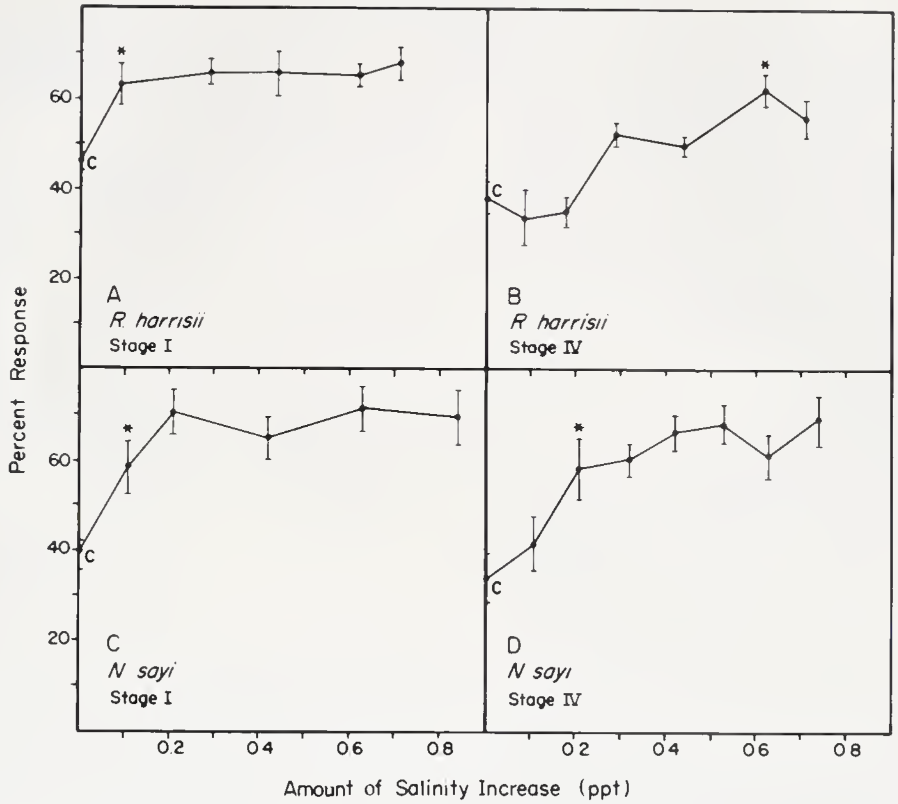

Figure 6. The percentage of *Rhithropanopeus harrisii* (A, B) and *Neopanope sayi* (C, D) larvae aggregating in the upper section upon exposure to the threshold rate of salinity increase (A, B = 1.1×10^{-3} ppt s^{-1}; C = 2.8×10^{-4} ppt s^{-1}; D = 7.0×10^{-4} ppt s^{-1}). Responses were measured after different absolute amounts of salinity change. *R. harrisii* larvae were acclimated to 20 ppt and *N. sayi* to 28 ppt. Five determinations were made for each zoeal stage except *R. harrisii* Stage IV zoeae where four determinations were made. Means and standard errors are plotted. An asterisk indicates the smallest salinity increase to induce a response significantly greater ($P < 0.05$) than the control (C) plotted at zero salinity change.

respond by ascending or descending, they are not exposed to a change in salinity during vertical movement. Similarly, when testing in sharp haloclines lower salinity water rests on top of higher salinity water. In this system larvae are either in one salinity or the other because the distance separating the salinities is small.

For the present experiments, there was a gradient of low to high salinity extending from the top of the experimental chamber to the bottom. In this situation larvae experience a continual increase in salinity upon descending and a continual decrease upon ascending. Thus if larvae respond to a salinity decrease and begin to descend, they will experience a salinity increase which evokes the opposite response. If there are differences in the threshold rates for responses to a salinity increase and decrease and the absolute amount of salinity change that must occur before a response, then only one directional response can occur in a gradient.

For example, the threshold rates for responses to a salinity increase are probably well below those to a salinity decrease for *R. harrisii* larvae. As for the absolute amount of change, responses of *R. harrisii* larvae to a salinity increase were clearly evident after 0.09 ppt for Stage I zoeae and 0.29 ppt change for Stage IV zoeae (Fig. 6). In contrast, Latz and Forward (1977) found that for both zoeal stages the threshold for step decreases in salinity ranged from 1 to 2 ppt. Thus *R. harrisii* larvae respond to both a slower rate of change and less absolute change upon a salinity increase in a gradient.

To explain the responses observed in the present experiments, one can assume that ascent and descent speeds of movement of *R. harrisii* larvae are similar (Latz and Forward, 1977). Now if the combination of larval upward movement and rate of salinity decrease in the test chamber becomes sufficient for a response to a salinity decrease, larvae will begin to descend. However, the rate of salinity increase experienced during a descent is well above threshold for a behavioral response and larvae move down until the absolute amount of salinity change (0.09–0.29 ppt) induces an ascent response. On

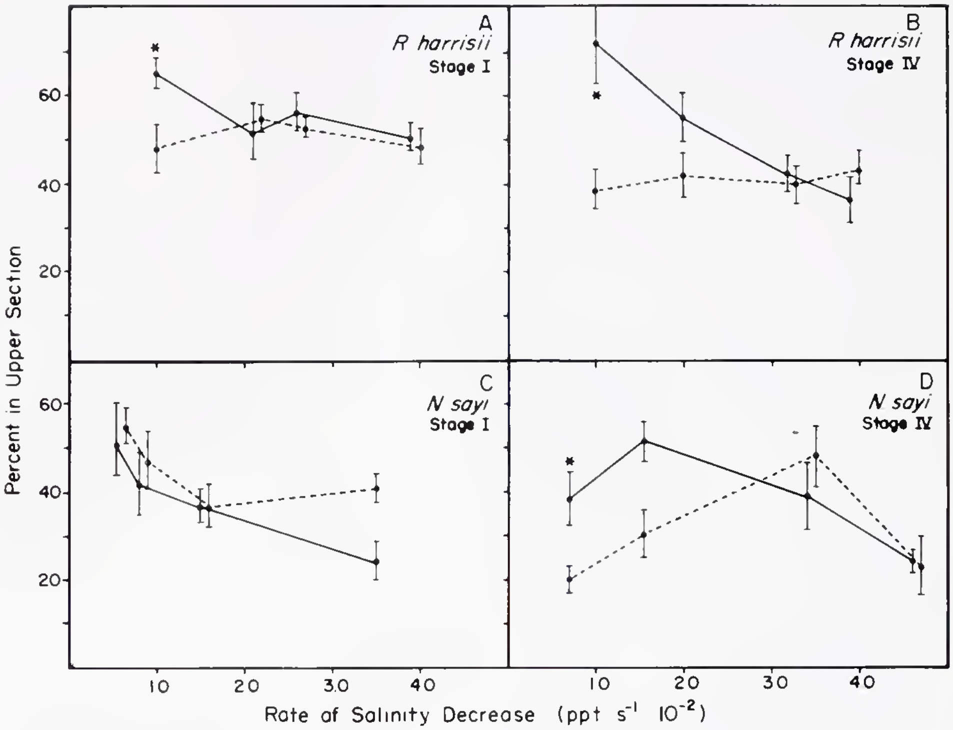

Figure 7. The percentage of *Rhithropanopeus harrisii* (A, B) and *Neopanope sayi* (C, D) zoeae aggregating in the upper section before stimulation (control—dashed line) and after 5.0 ppt change in salinity (experimental—solid line) at different rates of salinity decrease. *R. harrisii* larvae were acclimated to 20 ppt and *N. sayi* to 28 ppt. Five determinations were made for each rate and means and standard errors are plotted. An asterisk indicates rates at which the response is significantly ($P < 0.05$) greater than the control level.

ascending the rate of salinity decrease is sufficient to induce a behavioral response, but the absolute amount that salinity must change (1–2 ppt) before a response reversal is greater than upon descending (0.09–0.29 ppt). Thus larvae move up farther before the onset of a corrective response and should actually ascend upon a salinity decrease. This ascent response is evident in Figure 7 at the slowest rates of salinity decrease. At faster rates of salinity decrease, the salinity gradient in the experimental chamber became greater. Larvae display neither a descent (Figs. 4, 5) nor an ascent response (Fig. 7) and remained evenly distributed throughout the chamber. The absence of vertical movement occurred because the absolute vertical distances larvae moved during the descent and ascent responses probably became smaller in the steeper gradients.

Alternatively, upon a salinity increase, larvae respond to a slower rate of change than upon a salinity decrease. If exposed to a slow rate of salinity increase larvae will continually ascend in a gradient because the rate of salinity decrease encountered during the ascent is below threshold. The implications of these responses are that larvae should show responses to both increases and decreases in salinity at sharp haloclines as might occur after heavy rains, at fronts and in a two-layer estuary. Alternatively, larvae will ascend in small salinity gradients.

A consideration is whether crustacean larvae can respond to vertical salinity gradients in their environment. For *R. harrisii*, previous studies of responses to step changes in salinity (Latz and Forward, 1977) and of environmental salinity gradients (Cronin, 1982) suggest larvae should respond to environmental conditions. In contrast, a comparison of responses of *Callinectes sapidus* in haloclines with salinity levels in the larval habitat led Sulkin and Van Heukelem (1982) to conclude that the gradients are too small in nature to impede upward movement.

In the present study, responses to a salinity increase were most pronounced. For *R. harrisii* larvae, Cronin (1982) found a 1 to 8 ppt salinity difference between surface and bottom waters in a 3 to 4 m-deep water column at times of changing tidal flow direction in areas inhabited by the larvae. At other tidal phases, salinity was identical in surface and bottom waters. Assuming continuous salinity change from the surface to bottom, the predicted minimum and maximum salinity change ranges from

Table II

Calculation of the minimal detectable salinity change per m

Threshold rate salinity increase (ppt s^{-1})	Mean sinking rate (mm s^{-1})	Minimum detectable gradient of salinity increase (ppt m^{-1})
Rhithropanopeus harrisii		
Stage I—1.1×10^{-3}	3.1	0.35
Stage IV—1.1×10^{-3}	7.8	0.14
Neopanope sayi		
Stage I—2.8×10^{-4}	4.4	0.064
Stage IV—7.0×10^{-4}	6.2	0.11
Slowest test rate salinity decrease (ppt s^{-1})	Mean ascent rate (mm s^{-1})	Gradient of salinity decrease (ppt m^{-1})
R. harrisii		
Stage I—1.0×10^{-2}	6.3	1.6
Stage IV—1.0×10^{-2}	6.2	1.6
N. sayi		
Stage I—$.07 \times 10^{-2}$	3.2	2.2
Stage IV—$.07 \times 10^{-2}$	5.3	1.3

Rates are from Figures 2–5. Mean sinking (Latz and Forward, 1977) and ascent speeds (Forward and Wellins, 1988) for *R. harrisii* are at 20 ppt, while those for *N. sayi* are at 32 ppt (unpub. data). The minimal detection rate in ppt/m is calculated as (threshold rate/sinking rate)1000.

0.25 ppt m^{-1} to 2.7 ppt m^{-1}. A conservative measure of the speed of downward movement is larval sinking speed. If *R. harrisii* sink continuously, then the minimum salinity change they can detect is 0.35 ppt m^{-1} for Stage I zoeae and 0.14 ppt m^{-1} for Stage IV zoeae (Table II). Thus larvae can detect rates of salinity increase that are close to or slightly below the minimum rate of salinity change measured in their environment.

The minimum detectable salinity increases for *N. sayi* larvae are less than those for *R. harrisii* larvae (Table II) and range from 0.064 to 0.11 ppt m^{-1}. Measurements in lower estuarine (Williams *et al.*, 1967) and coastal areas of southeastern United States (Atkinson *et al.*, 1983; T. Johnson pers. comm. of salinity profile made from the R. V. Cape Hatteras along the North American east coast) suggest that sufficient vertical salinity gradients exist in areas inhabited by *N. sayi* larvae.

A response to a salinity increase requires not only a sufficient rate of increase but also an absolute amount of change. The minimum measured absolute amount ranged from 0.09 to 0.29 ppt for *R. harrisii* larvae and 0.11 to 0.21 ppt for *N. sayi* (Fig. 6). These amounts of change occur in areas inhabited by the larvae when gradients are present.

The minimum detectable salinity decrease gradient cannot be calculated because a descent response was not observed at any rate of salinity decrease. It is always possible that the foregoing explanation is incorrect and the absence of a response to a salinity decrease occurred because test rates were abnormally high. If larvae had responded to the slowest test rates of salinity decrease, then *R. harrisii* larvae could detect a gradient of 1.6 ppt m^{-1} and *N. sayi* gradient from 1.3 to 2.2 ppt m^{-1} (Table II). These values are within the levels observed in nature and thus are not abnormally high. The behavioral determination of detectable salinity decrease gradients may be impossible because of the extreme sensitivity to a salinity increase.

Considering the minimum detectable gradients of salinity increase, sinking rates (Table II), and the minimum absolute amount of detectable salinity increase, it is possible to calculate the descent time under these conditions before an ascent response occurs. It is assumed that larvae sink continuously. For *R. harrisii*, the times for Stages I and IV zoeae are 1.4 and 4.4 min, respectively, whereas the times for Stages I and IV zoeae of *N. sayi* are longer at 6.5 and 5.1 min, respectively. These times can be compared to rates of sensory adaptation to a change in salinity. Latz and Forward (1977) found that *R. harrisii* larvae showed total recovery to step changes in salinity in 5.5 min. Thus *R. harrisii* larvae can detect the minimum salinity gradients. If *N. sayi* larvae adapt at the same rates as *R. harrisii* larvae, then Stage IV zoeae of *N. sayi* can also detect the minimum gradient but Stage I zoeae requires either a slightly larger gradient or they must increase their descent rate by active swimming to prevent sensory adaptation from occurring before detection.

A further consideration is the functional significance of the asymmetrical responses to salinity change. They were extremely sensitive to rates of salinity increase but did not display the expected response to a salinity decrease. It is unlikely that the response to salinity increase is used to avoid adverse high salinity conditions. Larvae can usually develop in the highest salinities available in their environment. For example, *R. harrisii* larvae can complete development in salinities as high as 40 ppt (Costlow *et al.*, 1966). The real tolerance problem is to low salinity water (Forward *et al.*, 1982).

With the present study it is possible to evaluate similarities in responses of *R. harrisii* zoeae to rates of change in salinity, light (Forward, 1985), and pressure (Forward and Wellins, 1989). Upon descending in the water column salinity increases in a stratified system, light intensity decreases and hydrostatic pressure increases. At rates of change that are within the range a larvae can encounter while descending, each of these environmental changes induces negative geotaxis and/or an activity increase that results in an ascent.

In contrast, the opposite environmental changes upon an ascent produce weak responses at best. The present study indicated larvae do not descend in response to rates of salinity decreases they are likely to encounter in a salinity gradient. Larvae are unresponsive to rates of increase in light intensity that could be encountered underwater (Forward, 1985). In darkness, a positive geotaxis occurs to a pressure decrease but the threshold rate is much higher than that for a pressure increase (Forward and Wellins, 1989). Thus these asymmetrical responses of *R. harrisii* zoeae to environmental factors keep larvae up in the water column and reduce the likelihood that they will encounter the bottom and the associated benthic predators.

Acknowledgments

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Field Observations of Social Behavior, Shelter Use, and Foraging in the Lobster, *Homarus americanus*

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Abstract. Over a three-year period (1978–1981) behavioral observations of the lobster, *Homarus americanus,* were made by snorkeling in a shallow cove. Three hundred and thirty-four (334) animals were individually marked and this was the only time they were disturbed. In summer, the resident population numbered about 30 animals. The size composition, activity patterns, and habitat use of this population are described in a companion paper (Karnofsky *et al.,* 1989).

Shelters are of prime importance in the life of the lobster. Lobsters spent most of their time in shelters, leaving only at night. They dug shelters under eelgrass, rocks, and boulders; shelter locations appeared clustered. Some animals changed shelters frequently whereas others occupied the same shelters for up to 10 weeks. Premolt behavior was characterized by multiple shelter use. Cohabitation in the same shelter occurred only during periods of pair formation: when a mature female shared a male's shelter prior to and following her molt. We report the only field evidence for such courtship cohabitation.

Food foraging behavior was rare (0.35 instances/observation hour); most foraging involved live prey. Similarly, intraspecific interactions were surprisingly infrequent (0.2 instances/observation hour) and most, by far, did not involve physical contact. Although puncture wounds suggested intraspecific aggression, actual observations of escalating fights were rare. Premolt residents were involved in 65% of the interactions observed. In 70% of the interactions the larger animal won. However, smaller males and females could successfully defend their shelters against larger females. We report results from three homing experiments. The results suggest that much of the time that resident lobsters spend outside shelters is used to remain familiar with their constantly changing physical and social environment.

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Introduction

Most studies on individual behavior of the lobster, *Homarus americanus,* have been made under tightly controlled laboratory conditions and most were concerned with aggressive behavior, dominance, and shelter use (Scrivener, 1971; reviewed by Dunham, 1978; 1987; Atema and Cobb, 1980; Sastry and Ehinger, 1980; Finley and Haley, 1983). Controlled experimental situations are necessary when looking into behavioral mechanisms. However, such experiments cannot tell how relevant this behavior is in the behavioral ecology of a lobster. Without further knowledge of context the results from controlled studies cannot be interpreted. Feeding behavior of *Homarus americanus* has been observed in the laboratory (McLeese, 1970; Derby and Atema, 1982; Devine and Atema, 1982), but not in the field. Controlled and manipulative field studies on this species have addressed aspects of shelter use and population structure (Stewart, 1972; Richards and Cobb, 1986; Hudon, 1987). Some field studies have indicated possible territoriality in shelter use (Ennis, 1984).

Nonmanipulative studies in semi-natural environments in large aquaria circumvent some of the disadvantages of highly controlled environments and manipulative field studies. They have provided detailed knowledge about aggressive behavior, feeding behavior, courtship, pair formation, and the timing of molting and mating (Stein *et al.,* 1975; Atema *et al.,* 1979; Karnofsky and Price, 1989). However, even these conditions remain artificial, as it is impossible to imitate realistic emigration and immigration, the threat of predation, and the fluctuations in climate, shelters, and food. Controversies concerning the lobster's social behavior, such as the existence of a dominance hierarchy, territorial defense, or the incidence of escalated fights, and theories about mate selection, habitat utilization, and competition cannot be addressed without knowledge of behavioral relationships in the natural context. Only field studies can provide answers to these questions. To minimize human bias and misinterpretations due to human interference, a first study of this sort must be observational and nonmanipulative.

For such reasons we began a three-year (1979–1981) field study of *Homarus americanus*. The first year and a half we developed methods of marking and observation that would maximize the yield of data and minimize the interference with the animals' natural behavior. During the last 19 months we collected data in a standardized format. The results are reported in two papers, the first, a quantitative description of the population structure of lobsters found in this shallow habitat (Karnofsky *et al.*, 1989). This second paper is a description of the behavioral activities of that population.

Since the purpose of this behavioral study was to learn how lobsters live under natural conditions, undisturbed by human manipulation, we chose a site away from human activities and fishing: there is no lobstering in such shallow areas. For the same reason we limited our own manipulation to the absolute minimum necessary: individual marking of lobsters and their shelters. We were particularly interested in various aspects of shelter use, feeding, and social behavior. Because of our studies of courtship and pheromones in the laboratory (see Atema, 1986, for review) we were also interested in field evidence of courtship, male and female dominance orders, and mate choice strategies. These field data have allowed us a first glimpse of the natural behavior of this species. This information on behavior in an unrestricted population serves as a reference source against which results from manipulative field studies and controlled laboratory studies can be measured.

Materials and Methods

The study area was a shallow cove along a tidal channel on the southern edge of Buzzards Bay, Massachusetts (Figs. 1, 2). The substrate was divided between eelgrass beds, sandy spots, and an area called the "rock garden," containing small boulders covered with macroalgae. At low tide, water depth ranged from 0.3 to 1.5 m. Throughout the seasons water temperature ranged from 0 to 24°C.

All animals over 50 mm carapace length (CL) were marked with numbered claw bands and "pleural clips" (cut ventral tips of abdominal pleura) allowing recovery of both molt shells and molted animals. Bands were placed behind the dactyl so as not to interfere with claw use. Shelters were marked by placing numbered stones near them. To minimize interference with lobster behavior no other manipulation was performed.

The lobster population consisted of individuals ranging in size from 23 to 92 mm CL and most were subadults (<70 mm CL). The male-female sex ratio was approximately 2. Residents were defined as animals observed on more than one night; transients were defined as animals seen only during one night. During summer about 30 animals 50 mm CL and larger resided in the study area, while about 15 of these probably overwintered there. Lobsters, mostly smaller animals, were found even in areas with less than 0.5 m water depth. Turnover was considerable although some animals remained present for at least 13 months (Karnofsky *et al.*, 1989).

Observations were made during the nocturnal activity period using snorkel, a dim flash light, and a note sheet. Slow and gentle movements such as floating slowly over the study area using flippers or pulling ourselves along rocks caused no obvious changes in lobster behavior although occasionally the edge of a flashlight beam would startle them. Whenever possible we avoided shining the beam directly toward the lobsters. During most observations we scanned the area noting each animal's activities and location when encountered. The central study area was surveyed regularly (once or twice per observation night). Sometimes an observer would follow an individual animal for a longer period (a few minutes to an hour). Instances of cohabitation, agonistic interactions, and feeding were observed in more detail. Observation frequency ranged from 1–4 h per night, 1–6 nights per week, depending on temperature and visibility, totalling 333 hours of observation. Further details are given in Karnofsky *et al.* (1989).

Three homing experiments were conducted in September 1980. These were the only exceptions to the nonmanipulation rule. For these a battery pack with a periodically flashing, light-emitting diode was attached with cyanoacrylate glue to the part of the dorsal carapace that lobsters are unable to reach and groom with their walking legs. The two lobsters used were long-term residents with known and stable burrows. They were caught near their homes and brought to a boat dock where the pack was attached; this procedure took about 30 min. They were then released from the dock and followed by snorkeling without lights. For the first trials (7 Sept.) we used a 56-mm CL male who consistently occupied a shelter in the rock garden. For the second trial (16 Sept.) we used a 51 mm CL female whose shelter was in the same general area. For the third trial (23 Sept.) the male from the first trial who had lost his pack a few days after the first trial was again caught and refitted with a new pack.

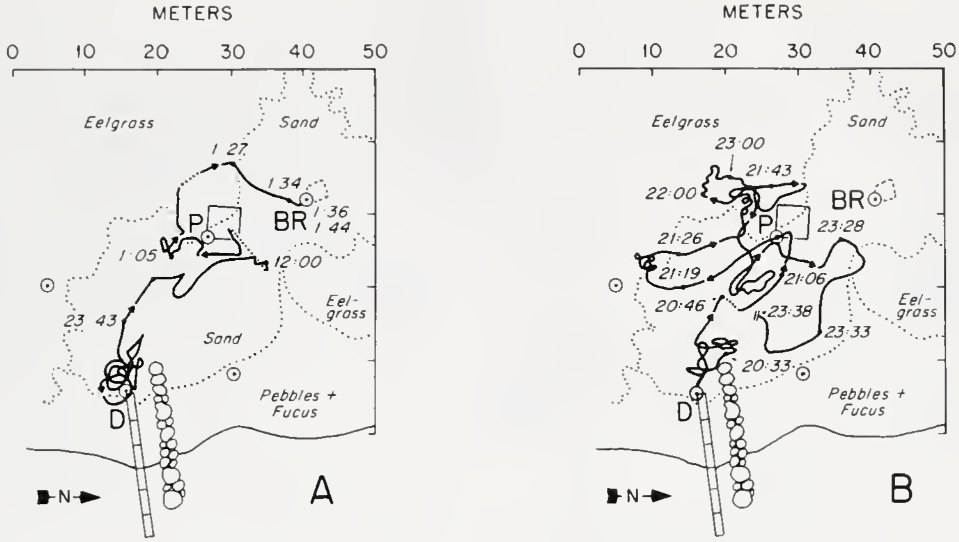

Figure 1. Detail of the field site map showing homing tracks of two lobsters, both stable residents of shelters near BR. A. On 7 September, a 56-mm CL male was released at the dock (D) at 23:24 h; he returned home (near BR) in 2 h, 12 min and remained there for at least 8 min, when observations were terminated; he continued to use his home shelter for several more weeks. B. On 16 September, a 51-mm CL female was released at D at 20:28 h; she did not return home (near BR) in the observation time (3 h, 10 min), but was found there the next day. She continued to be a stable resident in her home shelter. Dotted line: track lost. P indicates the 4 × 4 m chain link plot also seen in Figure 2. Circled points are fixed locations from which maps were drawn by triangulation.

Results and Discussion

In an attempt to determine if the animals were disturbed by our manipulations during marking, we compared the amount of time a lobster remained in the study site if it was caught at its shelter entrance *versus* caught when away from its shelter; we assumed that being caught near the shelter might pose a greater threat than being caught elsewhere. Disturbing the lobster near its shelter did not change the probability of its remaining in the study site nor the length of its stay (Chi-square, $P > 0.05$, d.f. = 369). In two cases animals involved in pair formation prior to mating (see *Courtship*) were caught and marked. In both cases the animals continued cohabitation without any obvious disturbance. Also, resident animals fitted with battery packs for homing studies continued to live in the same shelters. We concluded that our marking did not interfere seriously with lobster behavior.

Shelter use

In this shallow habitat, shelter appears to be critical since all animals spent much time inside shelter. During the day no lobsters were observed outside shelters. At night they were often seen bulldozing sand and debris out of their shelters, especially following storms. Eelgrass shelters almost always had two openings: one major entrance and a smaller "escape door." This has been seen in a variety of experimental and observational studies (Cobb, 1971). The number of entrances of rock shelters was more difficult to determine.

Lobsters would often barricade entrances to their shelters with a variety of objects, but particularly rocks, which they pushed or pulled with their (closed) claws, walking legs, and maxillipeds. Often such shelters would remain closed for up to two weeks. This may be related to molting as seen in the laboratory, where lobsters have been observed barricading their shelters for periods centered around their molt (unpub. obs.)

For the 66 molts recorded in the field we analyzed shelter-use in 2-week blocks prior to each molt date. The average number of different shelters occupied by a premolt animal increased steadily from 1, during the period 7–8 weeks before the molt, to 1.9 during the period 2 weeks before the molt. Moreover, during the early (7–8 week) premolt period, only 7 (10%) of the animals were seen in 3 or more shelters; this number increased to 21 (33%) during the 2-week period just prior to molting. The increased number of shelters frequented 1–2 weeks prior to molting may reflect increased aggression, in addition to increased activity seen in premolt lobsters (Tamm and Cobb, 1978; Atema *et al.*, unpub.). We speculate that spending time in several shelters may make a lobster's place of molt less obvious to competitors and discourage other lobsters from inhabiting the immediate vicinity.

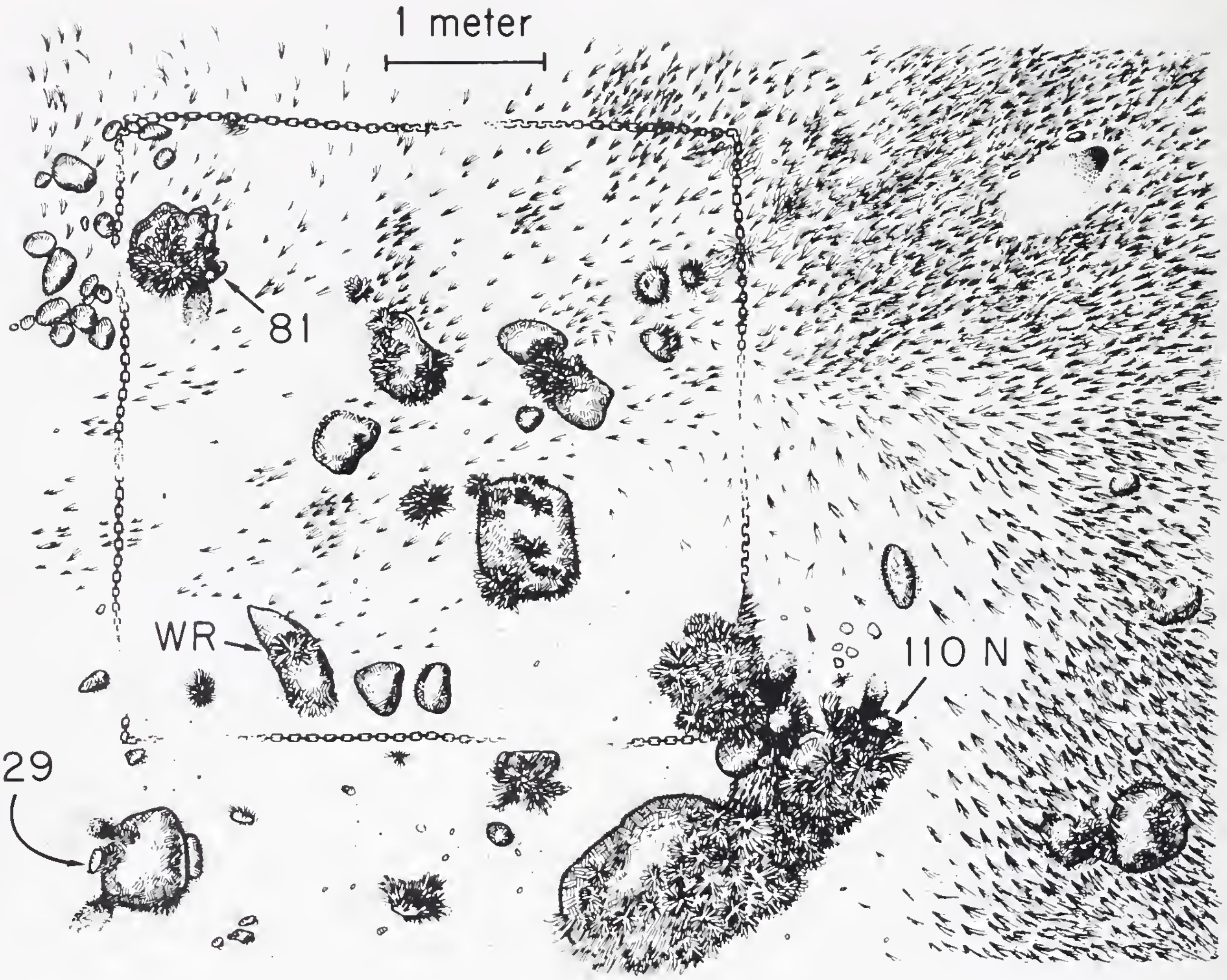

Figure 2. Further detail of field site showing lobster shelters dug in eelgrass (top right, unmarked) and under rocks. Many larger rocks were covered with macroalgae. Shelter #29 was a mating shelter in 1979; its resident male evicted the resident male of shelter #110N. Courting females often stood near WR from where they made approaches to #29. A lobster living in shelter #81 rarely emerged and did not interact with other animals. Chain marks area 4 × 4 m.

There were individual differences in shelter fidelity: some residents were rarely observed away from their shelters and occupied the same shelter consistently for several weeks or even months (n = 34), whereas others were never seen in any shelter (n = 26). Residents who were never seen in a shelter were also rarely seen in the site (an average of only 2–3 times); they must have had shelters just outside the observation area, because no animals were seen during the day. Ennis (1984) also reported differences in shelter fidelity.

Except for mated pairs, lobsters lived alone in shelters. Shelters appeared to be clustered (Karnofsky *et al.*, 1989: Fig. 1). Substrate features such as scars in the eelgrass, where it is easy to start digging under the roots, and rocky areas where shelters can be dug out under the rocks, may have encouraged clumping. Substrate features such as large boulders could also act as natural barriers setting apart shelter openings that were actually close in distance. The concentration of shelters in the rock garden could be explained by the large number of suitable rocks to burrow under. However, given that there were many rocks that were not used, and others that were used and then abandoned at some distance from the main shelter concentration, a social reason for shelter clumping seems possible. In the first figure in Ennis (1984) one can also observe shelter clumping, but it is unknown if this is related to habitat features or social behavior.

Lobsters seemed to have a good knowledge of their environment. When chased, as would happen occasionally for marking purposes, they would "tail flip" backwards directly toward and into a shelter opening. When the nearest opening was blocked by an observer the animals would tail flip into an alternative shelter or hiding place (this behavior has also been seen in the spiny lobster *Panulirus*). For example, the dominant male of shelter #29 could walk 5 m directly toward shelter #110N and evict the male (see Fig. 2). Females at WR (Fig. 2) walked directly to #29, 1.5 m away. Since accurate vision is unlikely (Fine-Levy, unpub. obs.), such observations imply that lobsters know their physical environment, as do various insects (Burk, 1988).

Homing

To gain further insight about the lobsters' knowledge of their physical environment, we conducted a few short-range homing experiments. Upon release from the dock, both the male (Fig. 1A) and the female (Fig. 1B) turned a few circles and meandered about the release site; both animals left in the general direction of their homes; both animals began to turn back in the area halfway to their home. The male turned a large loop through the eelgrass area and, upon leaving the grass, headed straight for home over sand. The female did not return home for over 3 h. The next night her back-pack was found detached but flashing in front of her home where she was also seen. The second time the male was released from the dock he went into a nearby shelter for 20 min and then disappeared; he could not be located despite an extensive search. The following night he was seen in the rock garden, having recently molted. On the third night the pack was found still attached to the molted carapace, flashing in front of his former home. Apparently the lobster had molted in his home shelter and was subsequently evicted (as is usual for newly molted lobsters in the laboratory, unpub. obs.). The next occupant then probably pushed the molt carapace out. In all three homing trials the lobsters returned to their home shelters within 24 h of capture, and probably between 2 and 6 h (*i.e.*, well before sunrise), since in this shallow site lobsters were never observed out of the shelter during the day. Our homing trials not only showed familiarity with the environment but also the importance of the home shelter: the male who molted within 24 h of the homing trial returned to his shelter for molting rather than staying in the closest unoccupied shelter.

Long-distance homing in *Homarus americanus* has been shown using tag-recapture methods (Pezzack and Duggan, 1986). Over a period of months lobsters were recaptured more than 100 km from the tagging site and then subsequently were captured again at the tagging site. However, these animals did not return to a particular shelter. Ennis (1984) described evidence for homing when a lobster that had been absent for some time was again seen in a burrow in which it had been seen earlier.

Foraging

Lobsters were seen searching for food as well as killing, carrying, and manipulating prey. However, considering the hundreds of observation hours, the number of food-related behaviors was surprisingly low. Over the 19-month study period, 117 instances of foraging were observed. Of these, 77 involved marked animals; the remainder were unmarked animals, mostly less than 50 mm CL. The proportion of males and females that were seen foraging (M63:F37) reflected the higher proportion of males in the population (Karnofsky *et al.*, 1989).

Foraging behavior was divided into four categories: (i) attacking or carrying live prey, (ii) carrying dead prey, (iii) carrying molt shell remains, and (iv) miscellaneous actions of unknown nutritional value. Live prey was much more common than dead prey. Of the 44 instances of attacking or carrying live prey, 14 attacks were not successful. Live prey items included snails (*Littorina littorea*), crabs (*Callinectes sapidus, Carcinus maenas, Ovalipes ocellatus,* and *Libinia* sp.), hermit crabs (*Pagurus* sp.), molluscs (razor clams, *Ensis americanus, Modiolus modiolus, Crepidula fornicata,* and *Placopecten irradians*), fish (sandlance, *Ammodytes* sp.), and polychaete worms (*Nereis vulgaris*). Preference for live prey was found previously (Squires, 1970; Weiss, 1970; Miller *et al.*, 1971). Lobsters were seen carrying dead prey in eight instances, including old fish carcasses used to bait traps and the remains of juvenile silversides (*Menidia menidia*) after bluefish attack. One lobster was seen manipulating a pork bone. Carrying molt shell remains, both of lobster and crab, was observed 19 times. Miscellaneous actions of unclear nutritional value were observed in 46 instances. These actions included digging, shell and pebble manipulation, probing with dactyls, and manipulations of algae and blades of eel grass.

Lobsters spent much time combing through various types of algae. They may have been gleaning small invertebrates. Although there is no strong evidence that they digest or feed on algae, lobster stomachs do contain algae (Herrick, 1911; Squires, 1970; Weiss, 1970; Ennis, 1973) and improved growth and survival of lobsters maintained in the laboratory have been shown when their diet includes small amounts of the alga, *Ascophyllum* sp. (Leavitt and Aiken, pers. comm.).

Some observations suggest that lobsters may actively hunt for buried sandlance. In fall, lobsters were seen feeding on sandlance that were half buried in the sand. Two weeks earlier, four lobsters had been seen digging large craters in open sand flats. The lobsters first dug down in the sand to a depth of about 10 cm and then continued to plow forward. Perhaps they were digging for buried fish that we could not discern.

Food burial as described by Smith (1976) and in naturalistic tanks (Atema and Leavitt, unpub.) was not seen during the study. Diet appeared to change with food availability. Lobsters continued to feed at low temperatures (5–10°C) well into the fall.

Agonistic interactions

Despite the lobster's reputation for being aggressive, only 71 instances of intraspecific interactions were observed (0.2 instances per observation hour). The interactions involved 52 animals, of which only 4 were transients. In most encounters, no physical contact occurred; only 5 (7%) included high level aggression such as "scissor, snap, attack, lunge" (definitions in Atema and Cobb, 1980) and 13 (18%) resulted in "tail flip." No wounds were inflicted. In a 30 m round pool tank with a population density similar to the rock garden, 6% of the interactions involved high level aggression (Karnofsky and Price, 1989). In a 7 m narrow tank with a higher population density, 17% of the interactive units recorded were high level aggression (Atema *et al.*, unpub.). In the same tank, Cowan and Atema (unpub. obs.) observed even higher levels of aggression when several mature males were present. The hypothesis emerges that most high-level aggression is related to the mature males attempting to establish a mating shelter and local dominance (see further evidence below and in following sections).

The ratio of males and females involved in agonistic interactions (2.2) was similar to the sex ratio (1.8) of the whole population. The number of *inter*sexual (19) and *intra*sexual (20) interactions were equal, and not significantly different (Chi-square, $P > 0.05$) from the expected value (22:17) given the sex ratio of the population. The larger animal won in 70% of all interactions. This has been well documented in laboratory experiments (review Atema and Cobb, 1980). Size-based dominance is characteristic for lobsters, but sex differences are important exceptions (see below).

Many interactions (24/71) involved animals defending their shelters, often successfully (16 successful defenses). Larger animals were usually successful in evicting smaller animals, but smaller ones were able to defend their shelters as well; in six (5M, 1F) of the nine instances in which smaller lobsters won a shelter defense, the smaller animal was defending its shelter against a larger female. In eight shelter approaches there were evictions (*i.e.*, the resident left and the challenging animal entered the shelter), usually between animals of the same sex; the winner was always the larger animal. Of the six evicted lobsters for which sex was known, four left the study area within a week of eviction. An equal number of males and females approached shelters (M8:F9). Males approached mostly males in shelters (M7:F1) which may reflect intrasexual competition in males (see *Courtship*). Females approached both sexes equally (M5:F4) in spite of the male biased population composition.

Naturalistic laboratory studies (Atema, 1986; Cowan and Atema, unpub.) suggest that male-female shelter interactions and evictions are the basis for intrasexual male dominance related to courtship. Based on the fact that in the field escalated fights were rare, we hypothesize that with few exceptions the 71 observed interactions were not direct dominance encounters, as suggested by staged laboratory fights (Scrivener, 1971), but served mainly to familiarize a lobster with its social environment. It stands to reason that social familiarity, as well as environmental familiarity (as described in *Shelter use* and *Homing*) would be advantageous particularly at the time of molting, both to avoid competition and predation, and, for mature animals, to secure mating partners. However, mature males may engage in dominance fights as observed in laboratory courtship studies (Cowan and Atema, unpub.; Karnofsky and Price, 1989). During this study we observed a male leaving shelter #29 (Fig. 2), running to a shelter 5 m away, and evicting its occupant, a large male. After evicting (presumably his competitor) he returned directly home. The evicted animal eventually returned to his shelter briefly, but subsequently left the area. In the laboratory such evictions are seen regularly. The eviction sequence itself has been described in detail (O'Neill and Cobb, 1979). These instances may be indications of territoriality or the expression of local dominance by one male (Kaufman, 1983; Karnofsky and Price, 1989).

Occasionally new lobsters were observed in a shelter originally occupied by another lobster. If the original occupant had not molted and was still in the study site, the second animal may have evicted it. In the rock garden, larger lobsters evicted smaller ones more often than the reverse (13:2) (Chi-square, $P < 0.05$). In the rest of the study area smaller and larger lobsters were equally likely (30:34) to be evictors. Thus, since lobsters show size related dominance, this may imply that the rock garden, but not other areas, contained preferred shelters. Rock shelters probably afford better protection and may be easier to defend. In addition, rock shelters are less modifiable than eelgrass shelters and thus properly sized rock shelters must be found and, if necessary, captured.

We observed a disproportionate number of interactions involving residents that molted in the study area (t-test, $P < 0.05$): 67 of the 103 interacting residents molted (65%), whereas only 6 of the 71 noninteracting residents molted (8%). Interactions may thus be related to impending molt. Since most interactions consisted of simple approach-avoidance behavior, this may reflect increased activity rather than increased aggression or both. Mature and subadult premolt lobsters in a semi-natural environment in the laboratory showed a distinct activity peak in the four days before their molt, and the dominant male showed increased aggression in addition to greatly increased activity (Atema *et al.*, unpub.). In staged fight experiments, a premolt aggression peak (during molt stages D_1 and D_2) has been shown for juvenile (10th and 11th stage) lobsters (Tamm and Cobb, 1978).

Based on these overall results, we hypothesize that high-level aggression is displayed by mature males establishing a mating shelter area, and that low-level aggression is generally related to premolt increase in activity.

Courtship

We observed four situations with evidence of courtship and pairing. Because data are rare and difficult to obtain (one cannot look into most shelters) we describe the observations in detail. Unfortunately, the most complete observations presented themselves in the preparatory phase of the field studies in 1979 before animals were marked with individual numbers. However, body size and left (L) or right (R) handed crusher (C) or seizer (S) claw can give partial identification.

In 1979 it was possible to look partially into shelter #29 (Fig. 2). We infer that the resident male of that shelter cohabited and mated with two females in sequence over a two-week period based on the following evidence. A banded resident male and an unbanded, one-clawed, left-seizer (LS) lobster were seen together inside this shelter occasionally "pushing" and "boxing," a common behavior for cohabiting pairs in the laboratory (Atema *et al.*, 1979; Atema, 1986). Two nights later an unbanded lobster with a normal seizer claw on the left side and a regenerating right crusher was seen with the same male in the same shelter. They were seen together for six nights. On the seventh night a two-clawed, banded, RC female was seen in the shelter with the same male. That same night the left seizer claw of a molt shell was found near the shelter. After two nights a large two-clawed, unbanded, RC lobster was seen with the male and stayed in the shelter for four more nights.

Laboratory observations in semi-natural conditions (Atema *et al.*, 1979; Atema, 1986; Cowan and Atema, unpub.; Karnofsky and Price, 1989) have shown that only mature males cohabit with mature females and then only for several days surrounding the female molt. In these laboratory studies dominant males retained their shelters during successive matings with different females. Therefore, we assume that both animals seen with the same male in shelter #29 were females. The molt remains of the left seizer claw and the sighting of an unbanded LS animal with a regenerating right crusher claw imply that the first LS animal was a female that molted during cohabitation. The subsequent sighting of a cohabiting, larger, unbanded RC lobster two nights after sighting a cohabiting banded RC female suggests that this second female also molted during cohabitation. Based on our laboratory observations, we infer that molting and mating occurred in both of these instances, and that the cohabitations of the one-clawed LS female and the two-clawed RC female with the same male followed each other almost immediately.

A number of additional observations showed that during this period the "mating" shelter #29 might have been unusually attractive to other lobsters. A small (<30 mm CL) lobster was seen regularly under an adjacent rock but was never approached by the inhabitants of shelter #29. A large male was seen digging at the back of the mating shelter; he subsequently approached the entrance, turned, and ran away. Two banded females (50 and 65 mm CL) were seen within 2 m of the shelter near WR (Fig. 2) on several consecutive nights. The larger made several approaches to the entrance of #29. The resident male appeared with a threat display (meral spread), stood outside and the approaching female left. Such shelter checking behavior by females is also common in laboratory observed courtship (Atema, 1986; Cowan and Atema, unpub.).

Some nights later a new banded male was seen in shelter #29. During the same night we caught a large newly molted male, perhaps the original male occupant, after he had approached the shelter and then fled. During the next few days several females, including the visitors mentioned above, inspected the shelter but cohabitation was not observed again. Subsequently, different males were seen in the shelter but none established long-term residence. Gradually fewer lobsters approached the shelter.

More indirect evidence for pair formation was obtained in 1980 and 1981. In 1980 a large, marked male (77 mm CL) and a new, initially unmarked female (80 mm CL) were seen in a shelter together. Two nights later the male was seen there again and subsequently a female molt claw and carapace were found in front of the shelter. The male left this shelter but remained in the area, where he was seen "bulldozing" in front of another shelter. The female was never seen again. In 1981 an initially unmarked male (77 mm CL) was caught and marked at a shelter where a marked female (61 mm CL) was also present. When he was returned after marking she stood near the entrance and both entered together. During subsequent observations they were seen together at the entrance. Two weeks later both were gone. The male was later seen elsewhere. In none of these latter cases could one look inside the shelters to gain more direct evidence for pairing.

While observations alone can never prove causality, observations in the field can provide both context and relevance for manipulative experiments in the laboratory and in the field. These observations of individually marked lobsters in their natural habitat provide valuable information on shelter use, foraging, homing, and agonistic behavior, and on cohabitation between males and females before and after the female's molt. These data are particularly useful because more detailed information was obtained from naturalistic environments in the laboratory. However, without the context of field studies, one must always doubt the validity of laboratory studies for natural behavior.

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Natural Dynamics of Population Structure and Habitat Use of the Lobster, *Homarus americanus*, in a Shallow Cove

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Abstract. We report the results of a nonmanipulative field study of the lobster, *Homarus americanus,* using long-term behavioral observations of marked individuals. We observed a freely mobile population in an open shallow cove habitat (50 m × 150 m) in Buzzards Bay, Massachusetts. Lobsters larger than 50 mm carapace length (CL) living in or entering the study site were marked individually (334 during the 19-month study). Without further manipulation, the animals were observed as long as they remained in the study site. Of the marked animals, 48% were transient, *i.e.*, seen only once.

The population was made up largely of subadults with a sex ratio of M:F = 1.8. The summer and fall resident population consisted of about 30 animals. Maximum residency was over 13 months. Half of the resident population, mostly small animals (50–59 mm CL), apparently overwintered in the site. A distinct peak in molting occurred both years in the spring at a water temperature of about 15°C. Injured animals were seen frequently (26% of the population) including a high proportion of mature resident males missing claws. Most other injured animals were transient (60%). These results suggest that the shallow cove is used as a refuge for injured mature males.

Activity was strictly nocturnal with a peak 1–3 h after sunset and declining through the night. Activity levels were equal for both sexes. Overall activity was correlated with seasonal variations in water temperature (0–24°C). At times, activity was correlated more with molting (premolt activity peak) than with temperature. Behavioral interactions in this population are described in a companion paper (Karnofsky *et al.*, 1989).

Introduction

The lobster, *Homarus americanus* (Milne-Edwards), is a slowly maturing, exceptionally long-lived, marine invertebrate inhabiting a large and varied geographic range from Newfoundland to Cape Hatteras, and from the low tide mark to the submarine canyons off the Continental Shelf at 400–600 m (Phillips *et al.*, 1980; Cooper and Uzmann 1980). This species is important both for scientific and commercial reasons. It supports a major fisheries in Canada and the United States and considerable effort has gone into its culture. Scientific investigations using lobster models range from studies on neurotransmitters, developmental neurobiology, and chemoreception to ecological models. Yet detailed knowledge of the natural behavior of *Homarus americanus* is sparse (reviewed by Atema and Cobb, 1980, Atema, 1986). Tag-recapture studies and submersible and SCUBA diver surveys provide some information about natural history, geographic range, and sex and age distribution in lobster populations (reviewed by Cooper and Uzmann, 1980; Campbell, 1986; Campbell and Stasko, 1986; Munro and Therriault, 1983; Pezzack and Duggan, 1986). Habitat may influence various aspects of behavior, population structure, and perhaps even social organization. Most field studies to date have relied on manipulations of either the population (Stewart, 1972; Hudon, 1987) or the habitat (with respect to artificial shelters, Scarratt, 1968; Sheehy, 1976) or both (O'Neill and Cobb, 1979). Such manipulations could affect the behavior of the population. Two previous studies in the field looked at long-term shelter occupancy: Stewart (1972) recognized residents, animals seen several times in a small area; Ennis (1984) found similar patterns and used the term territoriality to describe these types of habitat use. However, we

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prefer to use the commonly accepted definition of territoriality as defense of an area.

In 1978 we began a long-term field study in an area suitable for continuous, detailed observation throughout the seasons, restricting manipulation to marking individuals. There was no interference with the structure of the landscape. Since year-round observations of nocturnal marine animals are difficult, we chose a remote shallow cove where we could use snorkle rather than SCUBA to maximize data collection and to minimize disturbance by divers. In addition, the remoteness and shallowness of the site minimized interference from lobster fishing.

The aim of this study was to gain insight into the factors determining population structure and behavior in a society of unmanipulated, freely interacting lobsters. These field studies were complemented by laboratory studies in large naturalistic aquaria (Atema *et al.,* 1979; Atema, 1986). This paper describes the habitat, the structure of the lobster population, and the way it uses the shallow cove habitat; a companion paper describes the behavior of the lobsters in this population (Karnofsky *et al.,* 1989). Preliminary results of this study were reported in Karnofsky and Atema (1979).

Materials and Methods

The habitat

The field site was a shallow cove of about 50 m × 150 m along a tidal channel in Buzzards Bay near Woods Hole, Massachusetts (Fig. 1). Lobster shelters were found in water depths ranging from 0.3 to 1.5 m at mean low tide. The site experiences about 1 m of tidal fluctuation and a mean tidal current of about 1 km/h. The substrate consisted of mud and sand with eelgrass, bare sand, and rocks covered with macroalgae ("rock garden" Fig. 1). It sloped gently to a greater depth toward the channel. In winter and early spring the eelgrass was only about 10 cm long and sparse. Craters of 10 cm depth marked the location of collapsed eelgrass shelters from previous years. In various places in the eelgrass, furrows were gouged by ice flows. In the rock garden, sand was carried into shelter openings by winter storms. As summer progressed, the eelgrass grew to a length of 1–1.5 m. Their blades became a flat, impenetrable mat in the tidal currents, particularly at low tide. The channel connects two water basins, Buzzards Bay and Vineyard Sound, with different tidal cycles resulting in strong currents (up to 6 km/h) during times of high and low water. Along the channel the cove was delineated by an abrupt drop-off providing a bank, which was inhabited by larger lobsters. This area was not included in observations. Seasonal water temperature ranged from 0° to 24°C (Fig. 2A).

Data collection

Extensive preliminary studies were made in 1978 and 1979 to determine feasibility and to develop non-invasive methods. Observations with standardized procedures were made over 19 months, from 1 January 1980 to 31 July 1981, 1–6 times per week, depending on weather, and 1–4 h per night depending on water temperature and available observers, making up 333 hours of observation (Fig. 2B). For each observation period, tidal height, water temperature, underwater visibility, wind and wave action, and changes in flora and fauna were recorded. Observations were made almost exclusively by surface snorkling. Nearly all lobster activity was observed at night using hand-held, dim-white flashlights with narrow beams. Underwater notes were written with pencil on sanded pieces of plexiglass. Almost all observations were made between one hour after sunset and one hour before sunrise, with emphasis on the period of highest activity (1–3 hours after sunset; Karnofsky *et al.,* 1989). Observations were biased towards areas of higher lobster density and activity, but the entire area was searched at regular weekly intervals. High tide observations were difficult, especially on nights with poor visibility caused by storms and plankton blooms, and were therefore less frequent than low tide observations. Winter observations from January to March 1981 were nearly absent due to storms and ice.

All lobsters > 50 mm carapace length (CL) were caught once by hand or net, marked and described individually, and released at the exact site of capture a few minutes later. They were recaptured and rebanded after molting. During the 19 months of standardized observation, 334 lobsters were marked on the night of their first sighting. Marking consisted of placing one red and one white rubber band behind the dactyl on each claw where they do not interfere with claw use. Each band was printed with the lobster's identification number. A white band on the left clawmarked females, on the right, males. Preliminary observations had shown that this method of numbered, color-coded claw band marking was effective for quick identification of individuals, especially during nighttime observations, both inside and outside the shelters. Lobsters in shelters often had their claws showing at the entrance. In addition, the heavily calcified claws of a molt shell remained intact for several days (longer than other shell parts) and helped to determine an individual's time and place of molt. To estimate the date of a molt, information was combined about when, where, and in what condition the molt shell was found, the last day the animal was seen, and its carapace hardness when it was recaptured. Growth was determined by comparing the length of the carapace before and after molt.

To further mark each individual, we also clipped some

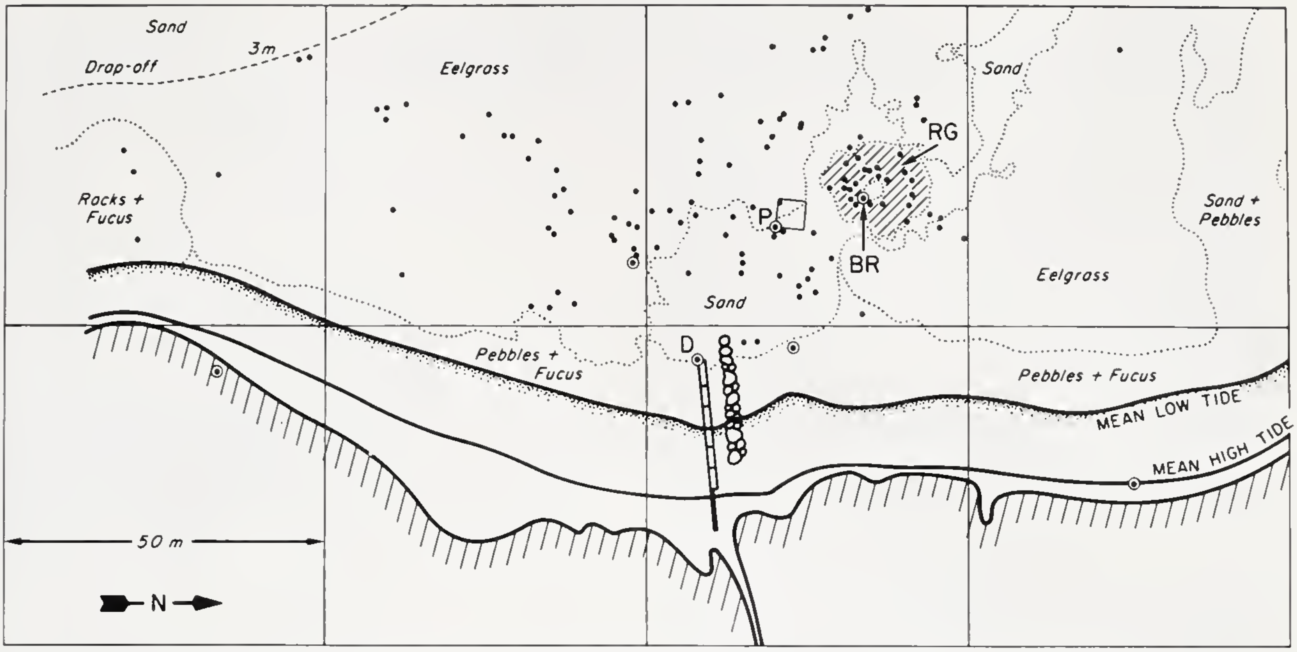

Figure 1. Map of field site showing substrate features and location of lobster shelters. Locations of prominent shelters and frequently used shelters are indicated with a dot. These shelters were individually labeled. D, dock; P, 4 × 4 m marked plot; BR, big rock; RG, rock garden (cross hatched). Grid 50 m. This map of shelter locations was drawn in 1979 using triangulation from fixed points on the shore and in the site (circled dots).

of the ten pleural points along the ventral abdomen in specific combinations. These clip marks remained visible for one or two molts. After 1–2 weeks of shell hardening, molted lobsters were re-marked with the same pleural clip code and a new band number. The description of marked lobsters included sex, size (CL in mm), left or right handed position of the crusher claw, exact time and location of capture, hardness of carapace, and other distinguishing characteristics such as missing appendages, scars, or growth of barnacles. Lobsters < 50 mm CL were marked periodically with a telson clip without regard to individual, sex, or size. Other interference with the lobster's behavior was avoided. During observations the location and activity of all marked lobsters were recorded. Unless otherwise stated, the group of individually marked lobsters larger than 50 mm CL is referred to as the "population."

The most frequently used home shelters were labeled by placing rocks with numbers painted on them next to the shelter opening, and the condition of these shelters was recorded regularly. New shelters were numbered as they appeared.

Results

Shelter

Approximately 130 shelters were marked during the study. Shelters were found either among small boulders (0.1–2.5 m diameter) where lobsters occupied crevices and holes repeatedly, or in the eelgrass burrowed under the roots. The presence of lobster shelters was usually revealed by a characteristic pile of freshly moved sand. Shelters often had two openings: one major entrance and a smaller "escape" door. Shelter openings were not oriented in any specific direction; for instance, they were not aligned with the tidal currents.

Residency

The individual lobsters making up the population of the cove were continually changing. Some lobsters were seen only once and considered "transient," whereas any lobster seen more than once was called "resident." Of the 334 marked lobsters, 174 were residents (52%) and 160 were transients (48%). The maximum residency period was greater than 13 months.

Not every resident was observed during each observation period; some were not seen for days or weeks. They may have escaped observation or left the area temporarily. To estimate the resident population, we adopted the following criterion for presence in the area. The mean and standard deviation of the longest gaps between sightings of each resident was 32 ± 9 days. Using this mean, plus one standard deviation, we considered a lobster to be present in the area if the sightings were not more than

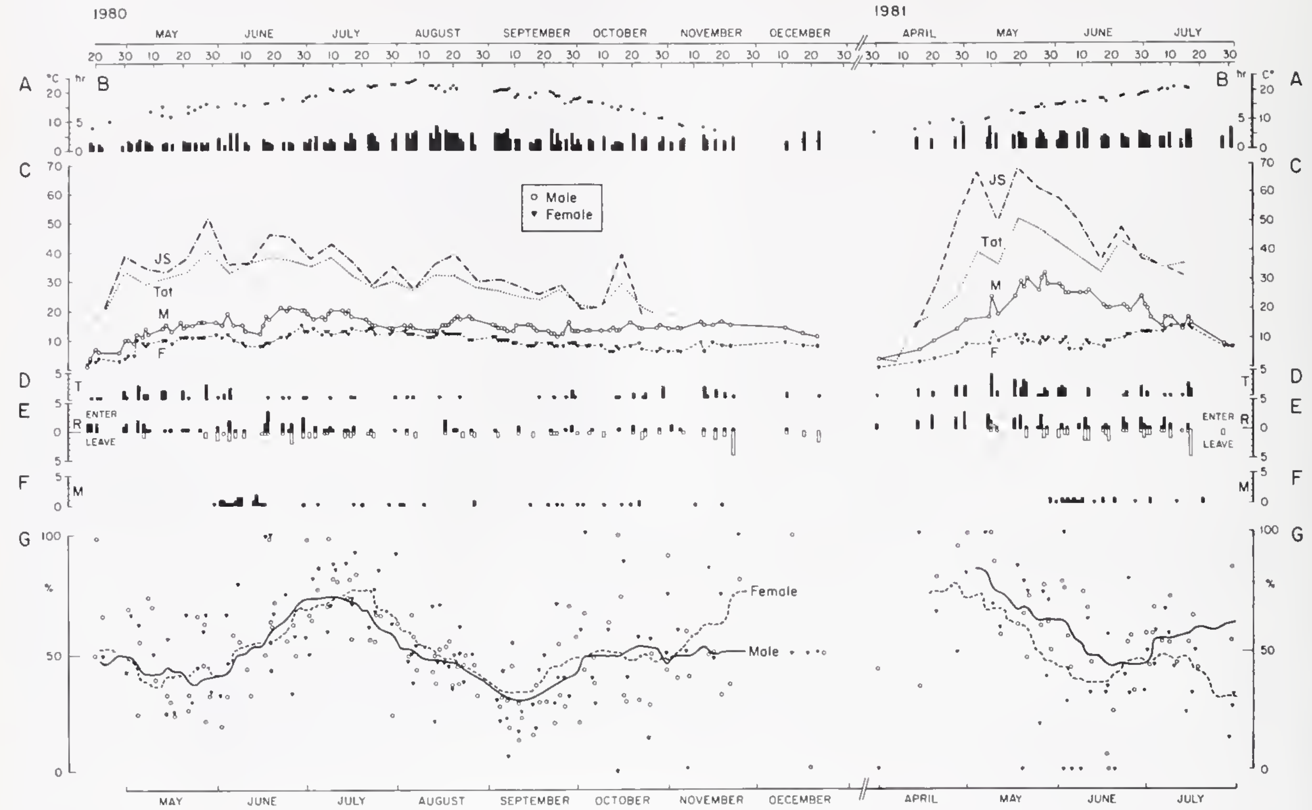

Figure 2. Seasonal description of daily lobster population. Due to weather, winter observations were sporadic and are not included in the figure. A. Water temperature (dots); left side of vertical scale indicates °C. B. Daily observation hours (bars); right side of vertical scale indicates daily number of observation hours. C. Population size: Calculations of daily number of males (M) and females (F) in the site (if an animal was seen within 41 days of its last sighting it was assumed to be a resident of the site, see text); calculation of weekly total number of all animals (Tot)—for this, each animal was counted only once per week; and Jolly-Seber estimate (JS) of weekly population size. *Note:* data after 20 June 1981 (*i.e.*, 41 days prior to the end of the study) are necessarily incomplete with regard to residency; thus population estimates are probably considerably below the actual number of animals present; some animals that were scored as "transients" (D) or "residents leaving" (E) would have been scored as residents if they had been observed again within a 41-day period after the end of the study on 31 July 1981. D. Daily number of transient lobsters (T). Transients were lobsters seen only the night of their initial marking. (See Note in C.) E. Daily number of resident lobsters (R) entering and leaving. Black bars upward: number of residents entering (the night of their initial marking); white bars downward: number of residents leaving (the night of their last sighting). Residents were lobsters seen during more than one night. (See Note in C.) F. Daily number of molting animals (M). Dates could be estimated, often precisely, from last sightings, post-molt recaptures and molt remains. G. Daily mean activity of males and females plus 28-day moving averages. Activity was calculated as the fraction of lobsters observed outside of shelter over the total number observed that day, based on daily first sightings of individuals only. ○ = Male, ▼ = Female.

41 days apart. Only 4 lobsters seen more than once had gaps between sightings longer than 41 days.

Based on these criteria for residency, the daily resident population size was determined separately for males and females (Fig. 2C; M, F). In addition, the total population per week was estimated (Fig. 2C; Tot). Weekly estimates were based on the total number of different individuals observed in the cove during one week regardless how many times an individual was sighted. This number cannot be derived from the addition of daily male and female totals, due to varying numbers of transients (Fig. 2D) and to residents entering and leaving (Fig. 2E). The resulting seasonal population curve of weekly estimates was closely approximated by weekly estimates calculated from the Jolly-Seber recapture model (Jolly 1965; Begon 1979) (Fig. 2C; JS). The Jolly-Seber model determines population size while taking into account transient animals. The population reached a peak of about 40 in June, of which about 30 were residents. The population then decreased gradually

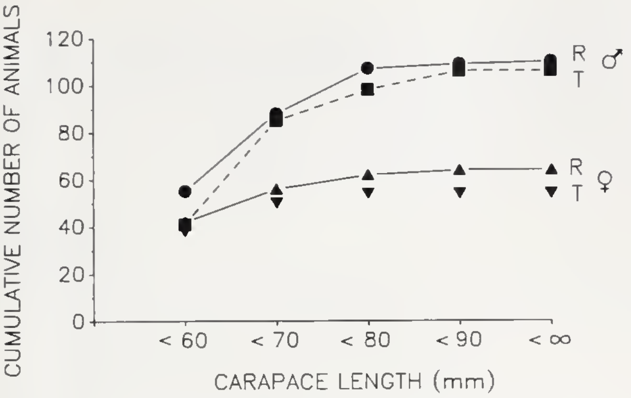

Figure 3. Cumulative size-frequency distributions for sex/residency groups based on size at which animals first entered the marked population (*i.e.,* not using their post-molt size). Smallest size class (<60) comprises animals 50–59 CL; next size class (<70) animals 50–69 mm CL, etc. Animals > 70 mm CL are considered mature. R = resident; T = transient.

through the summer and fall to about 15 residents in winter (see "overwintering" below).

Sex ratio, size distribution, molting, and growth

Throughout the year there were always more males than females (overall sex ratio M:F = 1.8). The sex ratios in the resident and transient group were similar (1.7 and 2, respectively) and did not change with seasons.

Size-frequency distributions were determined in 10 mm CL size classes for male and female residents and transients (Fig. 3); for lobsters of this size, 10 mm CL represents roughly one molt increment (see below). More than half of all marked lobsters were in the 50-mm CL size class (*i.e.,* 50–59 mm CL). Considering the overall sex ratio, females were over-represented in the 50 mm CL size class: this class contained 67% of all observed females but only 42% of all males (*t*-test, $P < 0.05$). Estimating that in the study area maturity both for males and females is reached between 70–80 mm CL (Estrella and McKiernan, 1986), 20% of the marked male population and only 10% of the female population was considered to be mature. The largest lobster was a 92-mm CL male. Thus, all calculations show that in this population the males were not only twice as common, but also relatively larger.

Sixty-six lobsters molted during the study period, 42 of them in a two-week period in spring of both years (Fig. 2F). We observed a peak of 23 molts between 31 May and 11 June 1980 and another peak of 19 molts between 29 May and 16 June 1981. In both years, in the month before the spring molt peak there was an influx of transients (Fig. 2D) and residents (Fig. 2E). Many residents left the area during and following the molt peak. There was no evidence of these animals molting a second time in the cove. Both the sex ratio and the size distribution of molting lobsters were similar to those of the total population.

Not all lobsters were recaptured after their molt. The mean premolt size of lobsters that remained in the site and were recaptured after molting was significantly smaller (57.4 mm CL for males; 58.2 mm for females) than the mean size of those that were not recaptured (64.5 mm for males; 63.3 mm for females) (*t*-test; $P < 0.05$). Only 3 of 28 animals in the smallest size class were not recaptured after molting, compared to 9 of 19 animals of 60 mm CL and larger. Twelve of 28 small animals and only 1 of 19 larger animals remained in the site for more than 25 days after molting; the difference is significant (G-test, $P < 0.01$). This indicates that after molting, larger animals leave the area sooner (or, less likely, that they become more elusive).

The average growth per molt was 9.6 mm CL (17% increase). There was no difference between males and females: growth per molt averaged 9.8 mm (17%) for males, and 9.3 mm (16%) for females. There was a significant seasonal difference in growth per molt (*t*-test; $P < 0.05$), with the largest increase observed in spring 1981 (11.7 mm; 19%). The smallest increment observed was 8% in a female of 51 mm CL (fall 1980), possibly caused by regeneration of a seizer claw.

Injury

Various minor injuries were seen: *i.e.,* puncture wounds, clipped or missing antennules and antennae, and missing walking legs. Ninety lobsters (27% of the marked population; Table IA) were missing one or both claws when first sighted, or, in seven cases, following their molt. Crusher claws were missing in 48 animals, seizer claws in 37, and 5 animals were missing both. The incidence of claw loss (Table IB) was greatest among large resident males (41%), followed by small transient females and males (38 and 35%, respectively). Claw loss was least frequent among large resident females (13%), followed by small resident males and females (17 and 18%, respectively). The difference in claw loss frequency between small (immature) transients and small (immature) residents is significant (G-test, $P < 0.001$), but the difference between large (mature) resident males and females is not significant (G-test, with Yates correction for small samples; $0.1 > P > 0.05$) (Table IA) probably due to the small sample size.

Activity

We determined lobster activity levels during the nighttime observations as did Ennis (1984), by dividing the number of times each individual lobster was seen outside

Table I

A. Incidence of claw loss: distribution of animals missing crusher claw (C) or seizer claw (S) or both claws (C + S), among resident (R), transient (T), male (M) and female (F) lobsters

	C	S	C + S	Totals		
R M	11	12	1	24		
R F	7	3	1		11	
						35R
T M	19	14	2	35		
T F	11	8	1		20	
						55T
Totals	48	37	5	59 M	31 F	= 90

B. Incidence of claw loss: distribution of claw loss (C, S, C + S) among small (immature) and large (mature), resident (R) and transient (T), male (M) and female (F) lobsters

		n/N	(%)	Total	(%)
Small (<70 mm CL)	R M	15/88	(17)	25/144	(17) **
	R F	10/56	(18)		
	T M	29/82	(35)	48/132	(36)
	T F	19/50	(38)		
Large (>70 mm CL)	R M	9/22	(41) *	10/29	(34)
	R F	1/7	(13)		
	T M	6/24	(25)	7/28	(25)
	T F	1/4	(25)		

n/N = Number of animals with one or two missing claws as a fraction of the total for the group.
** = Significant difference (G-test, $P < 0.001$).
* = Not significant (G-test with Yates correction for small samples; $0.1 > P > 0.05$).

a burrow by the number of times it was seen anywhere. The results were similar whether we used first nightly sightings of individuals or all nightly sightings. A 28-day moving average drawn through the points (Fig. 2G) showed no difference between the activity of females and males. Activity did not follow water temperature: decreasing activity in May and late July and August coincided with rising temperature, and increasing activity in October and November coincided with decreasing temperature. In 1980 the periods of low activity fell just before the spring and fall molt peaks; this was not the case in 1981. Although water temperature patterns in 1980 and 1981 were very similar, there were differences in activity patterns (Fig. 2G), indicating that temperature above 10°C did not predict activity as closely as found by Ennis (1984).

There was a pronounced circadian activity pattern. Lobsters were rarely seen during the day; they remained deep in their shelters. At sunset some began to appear at shelter openings, claws out. Most would not actually leave until about one hour later. The moving averages were used to determine whether nightly activity was related to time of night, and/or tide height or direction. Points falling above or below the moving average of Figure 2G indicate more or less than average activity. Above-average activity was more common during sunset observations (*i.e.*, 1–3 h after sunset) while below-average activity was more common during the less frequent sunrise observations (*i.e.*, 1–3 h before sunrise) (Table II). The correlation with time of night was significant: lobsters were most active between one and three hours after sunset. Generally they walked around slowly, checking shelters, interacting, and feeding. Activity decreased during the night with the lowest point being one to three hours before sunrise. Males and females, analyzed separately, behaved similarly. Although not statistically significant, a trend toward greater activity occured during the less frequent high tide observations and a trend toward lower activity during low tide observations. The direction of the tide, *i.e.*, rising or falling, showed no effect on activity.

Overwintering

During the few winter observations it appeared that lobsters hardly moved at water temperature below 5°C. One stiff-legged, barely walking animal was observed out of shelter in January 1980 at 5°C. About one half of the

Table II

Lobster activity: nightly and tidal changes

	Time of night			Tide			
	Sunset	Midnight	Sunrise	High	Low	Rising	Falling
Above avg.	43*	5	1*	15	35	32	23
Below avg.	25*	10	10*	7	42	20	28

* = Significant difference (χ^2, $P < 0.05$).

The number of points above and below the moving average of Fig. 2G are listed; each point represents the relative activity (%) of the population for that observation day, based on first sightings of individuals only (see text).

resident population (10 males, 5 females) were seen in late fall 1980 and again in 1981. In spring, 12 of these were in the same area (about 10 × 10 m) where they were last seen in late fall; 4 of these animals occupied the same 4 shelters before and after the winter. Two were males larger than 70 mm CL, and the remaining 13 animals were in the 50-mm CL size class. Nine of the 15 lobsters were observed well before the water temperature reached 10°C in April. The 15 lobsters probably overwintered in the cove. Later, 12 of them molted in the observation area. Five of these 12 had molted there in the previous fall.

Discussion

The data collected in this long-term study on a population of individually marked animals revealed several new and important aspects of the habitat use and population dynamics of *Homarus americanus.* These include long term residency, overwintering in shallow water, possible reasons for observed sex ratios, and a "refuge" function of the shallow habitat. It would be premature to generalize results which are determined at least in part by the habitat. Comparison with other habitats will be needed.

Although there was a steady stream of transient lobsters (as described by Stewart, 1972; Ennis, 1984), many lobsters were residents for considerable periods of time (months) in this shallow habitat. We had not suspected previously that so many animals lived in such shallow water (less than 1.5 m), let alone that they might overwinter there. It appears that at any one time in summer, at least 30 animals resided in the cove, concentrated in the rock garden and a few small peripheral areas of eelgrass. The weekly estimates were higher because they included individuals passing through the cove during the week. Although large lobsters were described in extremely shallow water during the 17th century (see Wood, 1967), lobsters, particularly large ones, are now rarely seen there. This may be due to fishing and/or increased human use of the shore line. The isolation of our shallow site may have contributed to its population density. However, perhaps the lack of daytime activity of lobsters in such shallow water has made them difficult to spot.

In winter, the number of residents appeared to decline to about 15. In keeping with the objectives of our study (*i.e.,* no disturbance except banding), we did not dig out overwintering lobsters to prove their presence. However, we consider the indirect evidence sufficiently strong to indicate shallow water overwintering by mostly small lobsters. Rock shelters are relatively safe from ice damage; overwintering in rock shelters may be an alternative preferable to the dangers of migration, such as exposure to predation and competition for shelters both elsewhere and upon return. Such dangers may apply particularly to smaller lobsters, which may not migrate as efficiently, and would suffer most from both predation *en route,* and shelter competition on arrival. Thomas (1968) described lobsters overwintering in 1.3–4 m of water in the mouth of the Bideford River, Prince Edward Island, Canada. Overwintering was common in Ennis' (1984) study, but the depth of winter shelters was not recorded and could have been down to 25 m.

A stable feature of the population was the male-biased sex ratio of about 2 (Fig. 3). The same ratio applied to various subpopulations, *i.e.,* resident, transient, molting, overwintering, and injured animals. Predominantly male populations were observed using trap statistics in shallow waters (4–10 m; Briggs and Mushacke, 1979) and in shallow artificial reefs (3–6 m: Zawacki, 1971; Briggs and Zawacki, 1974). In contrast, studies in deeper water (up to 24 m) have shown a 1:1 sex ratio (Krouse, 1973; Stewart, 1972; Cooper *et al.,* 1975) or even a predominance of females (Briggs and Mushacke, 1979; Estrella, 1983; Cooper *et al.,* 1975). The overall sex ratio of commercial catches is 1:1 (Cooper and Uzmann, 1980). The causes of the male-biased sex ratio in shallow water are not clear. We believe it could result from both behavioral-genetic and man-made factors. Natural lobster behavior could force mature males that lost competition for mating shelters (Atema *et al.,* 1979; Atema, 1986;

Karnofsky and Price, 1989) to move into more shallow areas; this fits with our observation of disproportionate claw loss of resident mature males in the shallow site and a limited number of mating shelters (Karnofsky *et al.*, 1989). For the same reason, maturing females may prefer to leave the area and move to deeper water because of the greater number of dominant males and mating shelters available for mating cohabitation. Mature females are protected by males during a cohabitation period surrounding their molt (Atema *et al.*, 1979; Atema, 1986). Deeper water might also protect female lobsters with externally carried eggs from storm-generated changes in salinity, turbidity, and temperature. In a Xanthid crab, Hazlett *et al.* (1977) found egg-bearing females in deeper water than females without eggs. In addition, since shallow waters are not fished, the relatively larger number of females in deeper water may be due to greater fishing pressure on males: deeper water is heavily fished, but egg-bearing females are protected from fisheries.

We noted that lobsters of all size classes moved into and out of the site. Our study only documented those larger than 50 mm CL. These animals could be recruited either from a resident population of smaller animals molting into the observed group, or from transient animals moving in from other locations. Both types of recruitment appear to occur. Some of the <50 mm CL lobsters that we telson-clipped without regard to sex or size subsequently molted into the observed population.

After the spring molt peak, all molted lobsters gradually left the study site. None of these animals molted a second time in the cove. Given the higher postmolt recapture rate for immature (<70 mm CL) lobsters, mature lobsters (>70 mm CL) may have left sooner after molting than the smaller ones. The general pattern of behaviors associated with the fall molt peak were similar to the spring molt peak except that not all lobsters left the cove following their molt. Of these, many were seen the following spring and some molted again in the cove during the spring molt peak before leaving.

A dramatic feature of the population was the large number of injured animals: 26% of the animals marked during the study were missing one or both claws. This was far more than the 15% claw loss determined in nearly simultaneous trap surveys of lobsters in Buzzard Bay (Estrella, 1983). Even in a Canadian habitat where algal harvesting caused general damage to lobsters, claw loss was only 14%, and this was double that of the normal incidence reported for that area (Scarratt, 1973). The great incidence of claw loss in our study suggests that this shallow site could serve as a refuge area for damaged animals perhaps escaping intraspecific competition.

Most animals with missing claws were small transients; however, among residents mature males missing claws were disproportionately represented (Table 1B). Similarly, Moriyasu (1984) reported that the percentage of lobsters missing claws increased over the summer in the Bideford River estuary reaching 30–40%, and that most of the large lobsters missing claws were males. Fishermen in that area suggest that lobsters missing claws move into the estuary ("hospital area") to regenerate. Since mature males compete for shelters that allow them to attract females (Atema, 1986; Karnofsky and Price, 1989) the losers of intrasexual competition may retreat to shallow water where competition for proper shelter is reduced. Lobsters missing one or two claws show increased tendency to flee when challenged (O'Neill and Cobb, 1979), but small animals rarely challenge large ones. Thus, because few large animals live there, the shallow habitat may serve as a refuge area for larger male lobsters missing claws. The smaller injured animals at our site were mostly transients. Since half the resident population in the cove was in this size class, this may reflect stronger competition between animals close in size as seen in staged laboratory fights (Scrivener, 1971). Similarly, brachyuran crabs of similar size show increased aggression (Sinclair, 1977) and when of markedly different size ignore each other (Vannini, 1980). However, social interactions are not the only causes for claw loss. Lobsters too small for commercial use can be damaged in traps before, during, and after they are released. Lobsters are also vulnerable following their molt, as illustrated by the seven lobsters newly missing claws after molting. Missing appendages may also be due to fish predation (A. Richards, pers. comm.).

Many lobsters molted during the first two weeks of June (Fig. 2F). This spring molt peak is probably the result of endocrine synchronization caused by the rise in water temperature to 10°C (Fig. 2A), coupled with increased photoperiod (Aiken, 1980). In both years, the number of transient lobsters increased during the month before the spring molt peak (Fig. 2D). These may have been lobsters looking for shelter in which to molt, but who were unable to establish residency due to intraspecific competition; both the overall population size had increased (Fig. 2C) and premolt residents may be more active and aggressive, as shown in laboratory studies (Tamm and Cobb, 1978).

In our marked, mostly immature population all lobsters had an equal probability of molting, and males and females grew equal amounts in a single molt. Our growth data were obtained from single molts per individual, unlike previous tag recapture studies which always lack direct information on the number of molts for a given individual. While we found no significant difference between male and female growth, most of these other studies indicated greater male growth (Ennis, 1972; Cooper and Uzmann, 1971; Squires, 1970; Wilder, 1953, 1963; Campbell, 1983); only Cooper (1970) found no difference in growth increments between sexes. The difference may be explained by sexual dimorphism in growth beginning to manifest itself at maturity (Templeman, 1935). Reduced growth in mature females may be caused by egg production (Squires, 1970). We would not see these effects in our study because most lobsters, particularly females, were immature, although female courtship behavior (Atema *et al.*, 1979) and ovarian development (review in Aiken and Waddy, 1980) may already begin in the 60-mm CL size class. In the population of our shallow site, males and females were almost equally represented among the 50–60-mm CL sized animals; however, females became increasingly less represented in the larger size classes (Fig. 3).

The shallow cove study site is only one of many different lobster habitats. Because of the shallow water depth, the elements (wind, sun, and ice) greatly affect the environment. In early spring lobsters could easily maneuver through the short eelgrass. Furrows carved out by winter ice provided starting places for burrowing under the eel grass roots. The rock garden was less affected by ice, but storm-induced sand-shifts often obstructed or uncovered the shelter openings. In summer the eelgrass grew into a dense mat, particularly impenetrable during the high currents at low tide. Many eelgrass shelters were abandoned and eels were often seen in the shelters unoccupied by lobsters. Such interspecific shelter competition may have contributed to the overall decrease of the lobster population over the summer (Fig. 2C).

In this shallow site lobsters were very rarely active during the day; they stayed deep in their shelters. In deeper water where the light level is lower, lobsters have been seen out of shelter during the day (Dr. Richard Cooper, pers. comm.). In our study, lobsters were most likely to be away from their shelters between 1 and 3 hours after sunset. Activity decreased during the night with a low point 1–3 hours before sunrise. This differs from a laboratory report of both post-sunset and pre-sunrise activity peaks (Cobb, 1969). Ennis (1984) found circadian activity peaking 2–3 hours after darkness, gradually dropping to very low before dawn and absent during the day. Similar results were reported by Cooper and Uzmann (1980). Our data also showed a tendency toward greater activity at high tide: in our site, the strong currents at low tide may hamper lobster movements and increase their vulnerability to predators.

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Ultrastructure and Development of Dimorphic Sperm in the Abyssal Echinoid *Phrissocystis multispina* (Echinodermata: Echinoidea): Implications for Deep Sea Reproductive Biology

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Abstract. Mature males of the abyssal echinoid *Phrissocystis multispina* produce two types of sperm including a euspermatozoon typical of echinoids, and a paraspermatozoon, which is bipolar-tailed. The structure of the testis and most features of spermatogenesis are similar to that of other echinoids. Development of both sperm types is identical until the spermatid stage when the nucleus of the paraspermatozoon undergoes chromatin reduction. Both sperm types have acrosomes typical of other echinoid sperm. However, we never observed a Golgi complex during any stage of sperm differentiation so the origin of the acrosome is unclear. Both the distal and proximal centrioles are involved in the formation of an anteriorly and posteriorly directed flagellum in the paraspermatozoon. Mixtures of both sperm types tend to clump due to the entanglement of sperm axonemes in the paraspermatozoon flagellum. Although the function of the paraspermatozoa is unknown, they may play a role in facilitating fertilization through the reduction of euspermatozoon diffusion during spawning. This study reports only one of several recently discovered reproductive adaptations associated with deep-sea habitats.

Introduction

Echinoderms are often the most abundant and diverse macrofaunal organisms in the abyssal and bathyal zones of the ocean (Billet and Hansen, 1982; Pawson, 1982) and the reproductive cycles of many deep-sea species have been investigated (see Lightfoot *et al.*, 1979; Tyler *et al.*, 1982; Tyler, 1988). Until now, however, technology has restricted the study of deep-sea reproduction largely to the analysis of paraffin sections of preserved gonads collected from trawls or dredge collections (George and Menzies 1967, 1968; Rokop, 1974, 1977; Ahfeld, 1977; Pain *et al.*, 1982a, b; Tyler and Gage, 1982, 1983, 1984; Tyler *et al.*, 1982). Little is known about gamete structure, fertilization biology, or development of any deep-sea species due, in part, to the difficulty of obtaining live specimens in good condition.

Echinoderm sperm have been extensively studied and, in comparison to most invertebrate sperm, are morphologically conservative (Chia and Bickell, 1983). This study describes the ultrastructural features of spermatogenesis in *Phrissocystic multispina,* a deep-sea spatangoid collected in Hawaiian waters with the Pisces V submersible. Males of *P. multispina* have dimorphic sperm, the first recorded occurrence in the Echinodermata, and although one type of sperm is typical of echinoids, the other is bipolar-tailed, a feature which is structurally rare among metazoan sperm.

Animal sperm morphology is strongly influenced by the mode of fertilization and the environment into which the sperm are released during spawning (Franzen, 1956, 1970). The appearance of dimorphic, aberrant sperm in *P. multispina* and unusual sperm in several other deep-sea echinoderms (Healy *et al.*, 1988; Eckelbarger *et al.*, in press) suggests that unique selective pressures may be present in the abyssal zone of the deep sea which are absent from shallow water habitats. The present paper describes the ultrastructural features of spermatogenesis in this species and discusses the potential factors influenc-

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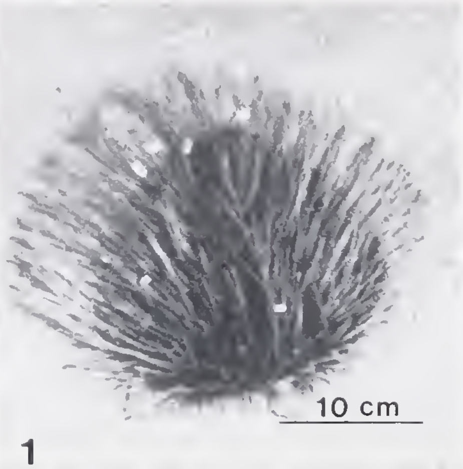
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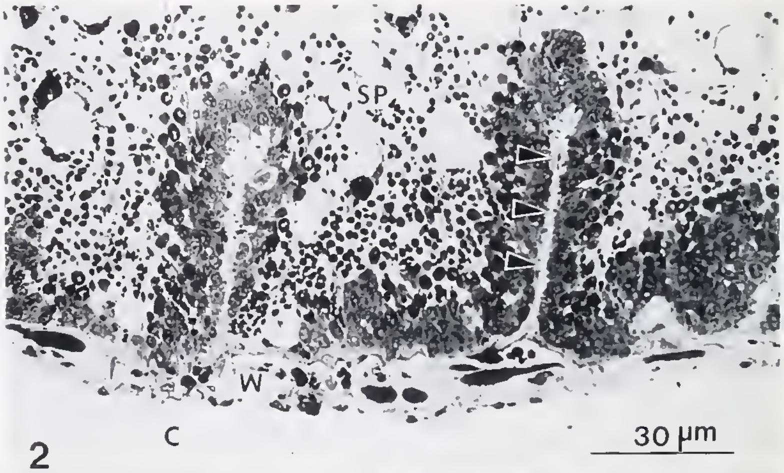
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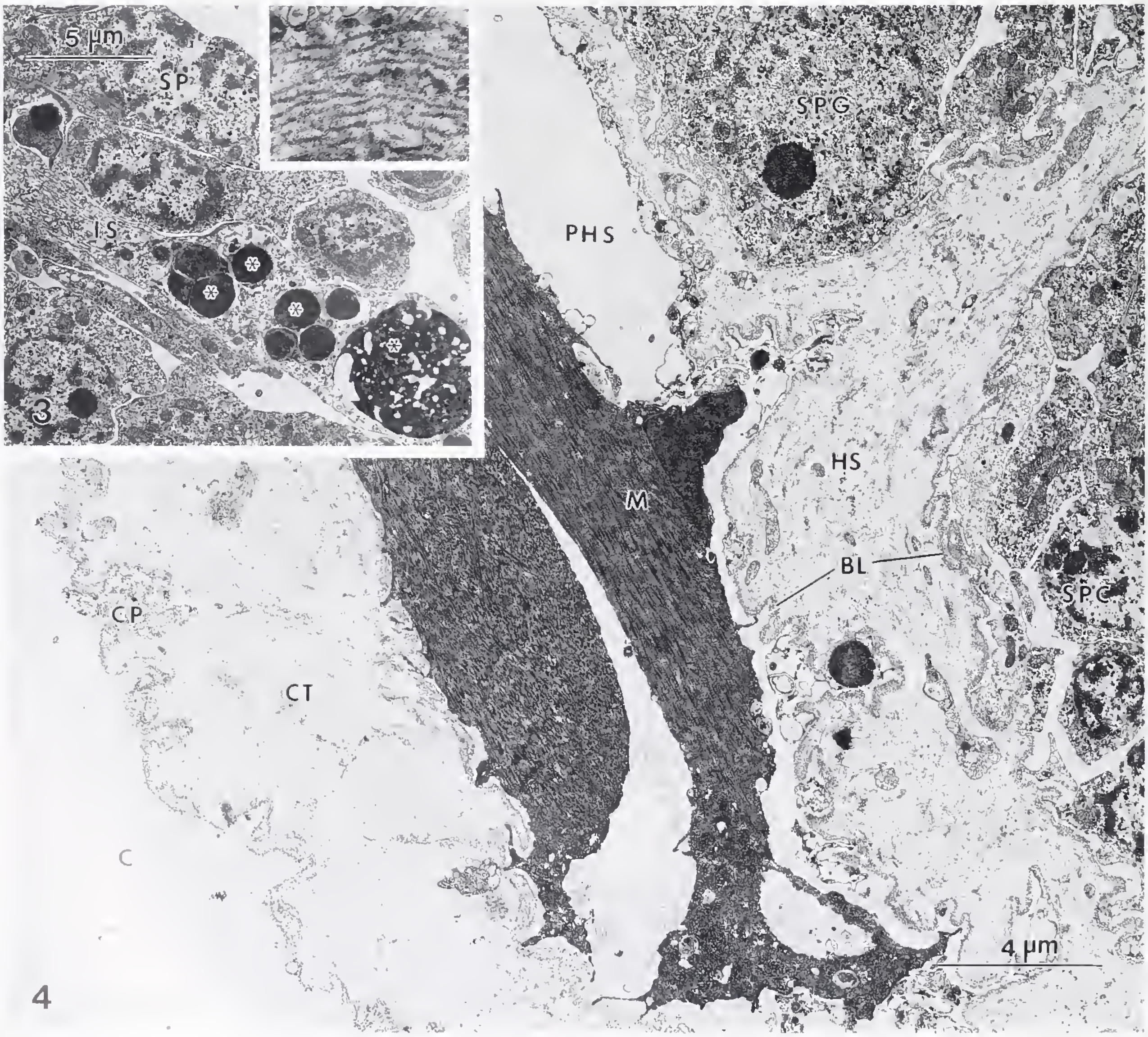
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ing the evolution of these unusual gametes. This investigation represents only the third study of gamete ultrastructure in a deep-sea organism. The ultrastructure of sperm development in the vestimentiferan, *Riftia pachyptila,* was previously described from specimens collected near hydrothermal vent communities using the manned submersible Alvin (Gardiner and Jones, 1985). Recently, aberrant sperm were reported in *Xyloplax,* a deep sea representative of the new echinoderm class, Concentricycloidea (Healy *et al.,* 1988).

Materials and Methods

Six live, sexually mature specimens of *Phrissocystis multispina* (four male and two female) were collected off Kailua-Kona, Hawaii, in July 1988 at depths ranging from 875 m to 1920 m using the Pisces V submersible (Fig. 1). Specimens were dissected on board ship shortly after collection and testes were removed for immediate fixation. Whole testes were fixed for 1 h in a primary fixative (2.5% glutaraldehyde in 0.2 *M* sodium cacodylate, 0.1 *M* NaCl, and 0.35 *M* sucrose at 4°C) and then washed in three changes of 0.2 *M* sodium cacodylate, 0.3 *M* NaCl, and 0.35 *M* sucrose. Tissue was then postfixed in 1% OsO_4 in 0.2 *M* sodium cacodylate, 0.3 *M* NaCl, and 0.35 *M* sucrose at room temperature. Tissues were then dehydrated in ascending concentrations of ethanol, transferred through two changes of propylene oxide, and embedded in Epon. Thick sections were cut on a Porter-Blum MT2-B ultramicrotome using a diamond knife, mounted on slides, stained with Richardson's stain, and photographed with a Zeiss WL research compound microscope. Thin sections were cut with a diamond knife and stained with alcoholic saturated uranyl acetate and aqueous lead citrate for 10 min each, then examined in a Zeiss EM9S-2 transmission electron microscope.

Dried specimens of *Phrissocystis multispina* have been deposited with the U. S. National Museum, Smithsonian Institution (USNM # E 37821).

Results

Structure of the testis wall

The testes are arborescent structures suspended by folds of the perivisceral epithelium from the interambulacral plates in the apical half of the perivisceral coelom. A cross section through a single acinus of the testis reveals a thin, apparently unflagellated outer perivisceral epithelium, followed by a thick (3.5–4 μm) collagenous connective tissue layer, a narrow perihemal sinus lined by a thin basal lamina and containing prominent muscle cells, a hemal sinus about 2 μm in width, and the testis lumen lined by a germinal epithelium (Fig. 4). The hemal sinus periodically evaginates into the testis lumen creating columns of developing sperm cells (Fig. 2). Elongated interstitial cells (Fig. 3) are often observed in association with developing germ cells and contain a variety of spherical electron-dense granules and numerous, parallel microtubules arranged in the long axis of the cell (Fig. 3, insert).

Spermatogonia and spermatocytes

We observed two morphological types of sperm in four male specimens of *Phrissocystis multispina.* The two types appear in approximately equal proportions, based on squashes of fresh testes and examination of histological sections. Due to extensive injury to specimens during submersible collection, we were unable to observe spawning in intact animals. One spermatozoan type is "typical" for echinoids while the second is bipolar-tailed and "atypical" in morphology. Limited observations of living sperm indicated that both types were motile but sluggish swimmers. Various terms have been suggested to describe polymorphic sperm based on differences in nuclear chromatin (Meves, 1903; Melone *et al.,* 1980; Buckland-Nicks *et al.,* 1982). However, the two types of sperm observed in *P. multispina* appear to have identical nuclear morphology. To avoid the proliferation of new terminology, we have adopted the terms "euspermatozoon" for the typical sperm and "paraspermatozoon" (para: near, close) for the atypical sperm as proposed by Healy and Jamieson (1981). Differentiation of these sperm cannot be distinguished until the early spermatid stage so earlier development will be described for both simultaneously. Spermiogenesis will be described separately for each sperm type.

Examination of the germinal epithelium reveals the presence of spermatogonia and spermatocytes, while the

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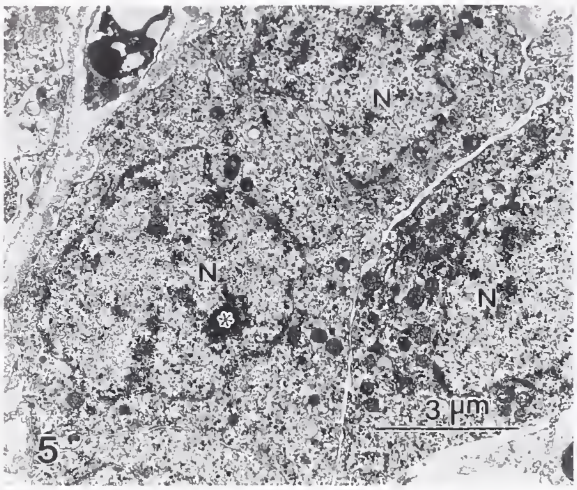
Figure 1. Adult specimen of *Phrissocystis multispina* photographed from the Pisces V submersible at a depth of 1920 m off Kailua-Kona, Hawaii.

Figure 2. Light microscopic transverse section through the testis wall. Arrows indicate invagination of hemal sinus into testis lumen. C, coelom; SP, sperm cells; W, wall of testis.

Figure 3. Interstitial cell (IS) containing electron dense inclusions (*) surrounded by developing sperm cells (SP). Insert: higher magnification of interstitial cell cytoplasm showing parallel arrays of microtubules.

Figure 4. Transverse section through the testis wall showing the outer coelomic peritoneum (CP); connective tissue layer (CT); large muscles cells (M) within the perihemal sinus (PHS); the hemal sinus (HS) lined by a basal lamina (BL) and adjacent to a germinal epithelium containing spermatogonia (SPG) and spermatocytes (SPC). C, coelom.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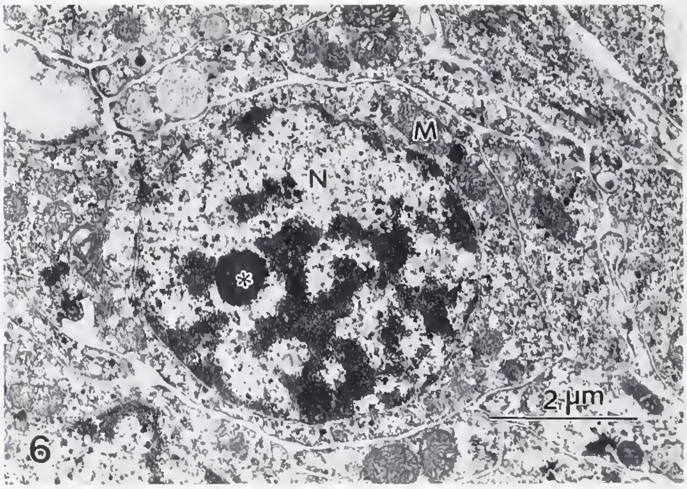
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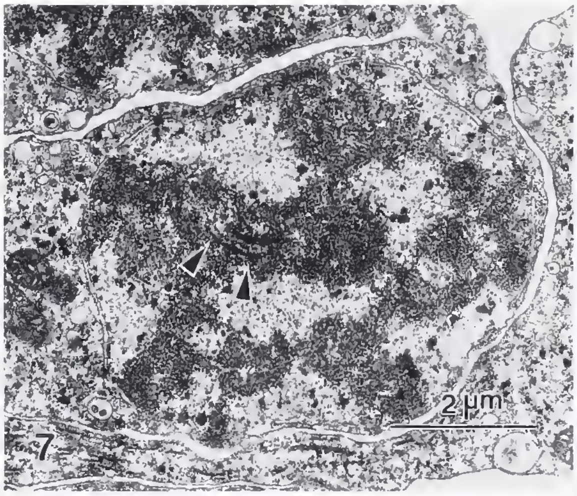
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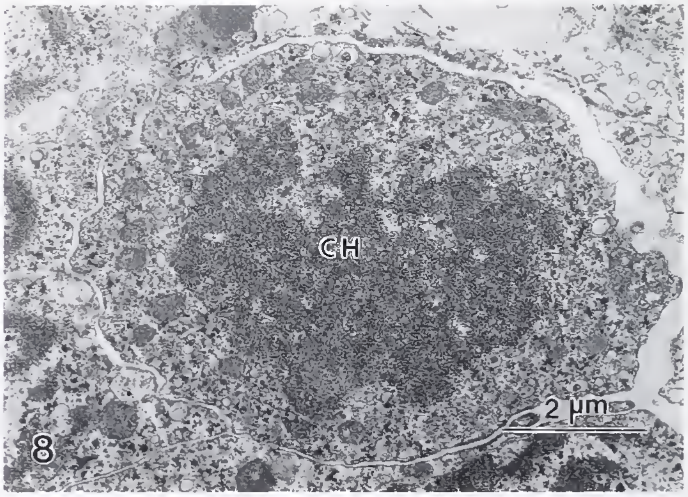
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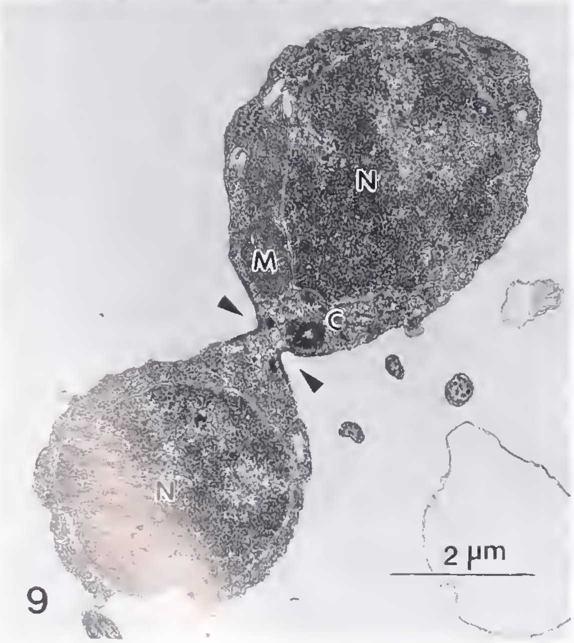
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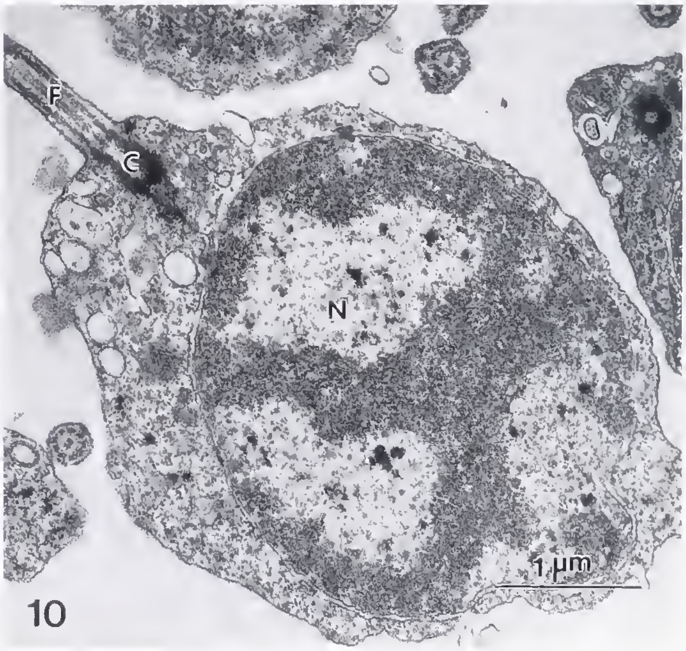
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lumen of the testis contains mostly spermatids and mature sperm. Spermatogonia lie closest to the hemal sinus (Fig. 4) and are the largest and least abundant type of germ cell (Fig. 5). They are roughly spherical in shape and contain a large (7.0–8.0 μm) nucleus, a few circular profiles of mitochondria, and numerous free ribosomes. The nucleus contains a fine granular chromatin and usually a small single nucleolus. Spermatocytes in the preleptotene/leptotene stage of first meiotic prophase are the most common germ cell and are generally spherical cells with smaller (5.0–6.0 μm) nuclei each containing a prominent nucleolus and scattered patches of electron-dense heterochromatin (Fig. 6). Occasionally, a spermatocyte in the zygotene/pachytene stage of first meiotic metaphase is observed with characteristic synaptonemal complexes (Fig. 7). Spermatocytes in the process of mitotic division are relatively common (Fig. 8).

Spermiogenesis—euspermatozoan

Spermatids are small spherical cells with round nuclei about 2.5–3 μm in diameter that are interconnected by intercellular bridges (Fig. 9). In thin sections, as many as three spermatids have been observed in cytoplasmic continuity. Bridges are characterized by electron-dense plaques along the inner plasmalemma. Spermatid nuclei have regions of electron-opaque chromatin surrounded by a finely condensed background. Organelles are sparse and include a few small mitochondria and a pair of centrioles that lie near the intercellular bridges, thus initiating a cytoplasmic polarization (Fig. 9). The distal centriole forms a single flagellum that projects from the future caudal region of the cell (Fig. 10).

Spermatids break their intercellular connections and float freely in the testis lumen when the nuclear chromatin becomes equally distributed throughout the nucleus and granular in texture (Fig. 11). A shallow centriolar fossa appears at the base of the nucleus and an electron-dense plaque forms along the inner surface of the nuclear envelope. The proximal centriole lies perpendicular to the long axis of the cell and bears a fibrous appendage that extends from its proximal surface into the centriolar fossa (Figs. 11, 12). The distal centriole and the spermatid flagellum lie posterior to the proximal centriole.

As differentiation continues, the entire cell and its nucleus elongate (Fig. 12). The mitochondria have now presumably fused because only a single large one remains. It is positioned in the future middlepiece region of the spermatid and surrounds both centrioles (Fig. 12). A small proacrosomal granule appears abruptly at the future apical pole of the spermatid in close association with the nucleus (Fig. 13). It consists of a small sac containing a fine granular product, located between the outer nuclear envelope and the cell plasmalemma. A Golgi complex was never observed in developing sperm cells at this or any other stage despite examination of numerous semi-serial sections. However, serial sectioning of sperm stages was not undertaken.

Further differentiation results in a mature spermatozoon (Figs. 14, 15) with a head length (middlepiece, nucleus, and acrosome) of about 4.5 μm. The nucleus is about 4.0 μm in length and 2.0 μm in width, and bears a small, cap-like acrosomal vesicle measuring about 300 nm in length and 275 nm in width. The vesicle is subspherical with a flattened basal surface (Fig. 17). It contains a fine granular product and the inner surface of the posterior acrosomal membrane bears an electron-dense plaque. A small subacrosomal fossa forms a depression at the anterior tip of the nucleus and contains fine, reticular periacrosomal material. The middlepiece is symmetrical with centrally placed centrioles at right angles to each other surrounded by a single, circular mitochondrion (Fig. 16). The distal centriole rests in a shallow centriolar fossa at the base of the nucleus (Fig. 14). The flagellum exhibits the 9 + 2 pattern (Fig. 14, insert).

Spermiogenesis—paraspermatozoan

Due to the limited amount of material available for ultrastructural study, we were unable to conclusively elucidate all stages in the development of this sperm type, particularly with respect to the differentiation of the flagellum.

Early spermatids resemble those of the euspermatozoa in most respects except for the appearance of two axonemes, one of which remains intracellular. Only two centrioles were ever observed, and to avoid confusion, the centriole producing the primary flagellum used in propulsion will be designated the distal centriole. The proximal centriole forms a secondary axoneme which remains intracellular and closely associated with the nucleus throughout spermiogenesis.

Figure 5. Three spermatogonia with large, irregularly shaped nuclei (N) and small nucleolus (*).

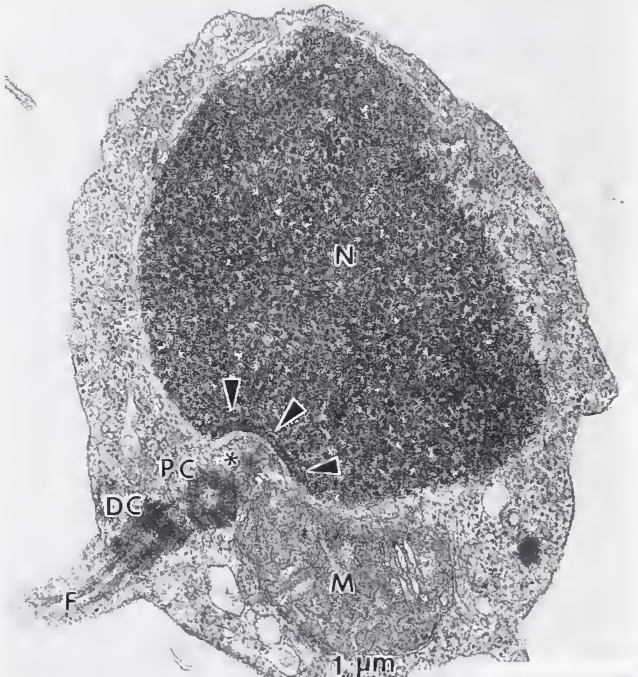
Figure 6. Spermatocyte with large circular nucleus containing a small nucleolus (*). M, mitochondrion.

Figure 7. Spermatocyte nucleus containing synatonemal complex (arrows).

Figure 8. Spermatocyte in process of mitotic division. Note dispersed chromatin (CH).

Figure 9. Two spermatids connected by intercellular bridge (between arrows). N, nucleus; C, centriole; M, mitochondrion.

Figure 10. Spermatid with large nucleus (N), posterior flagellum (F) and associated centriole (C).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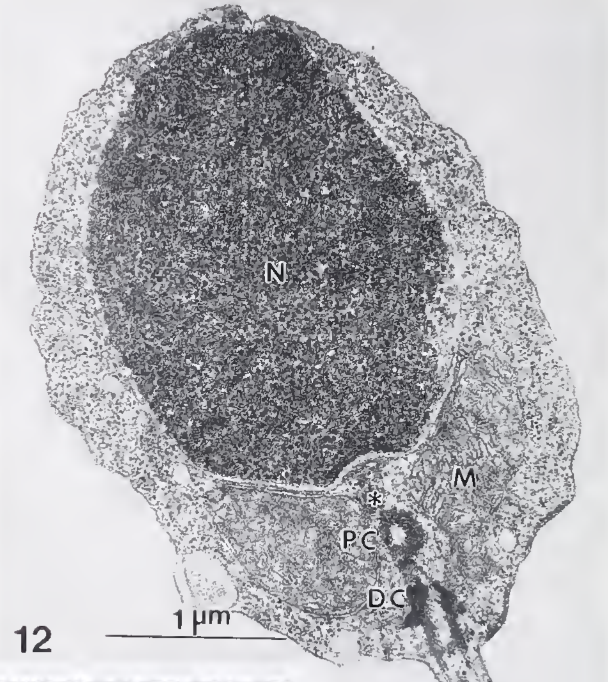
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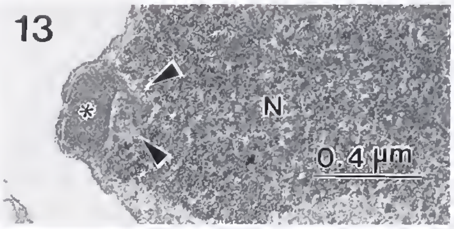

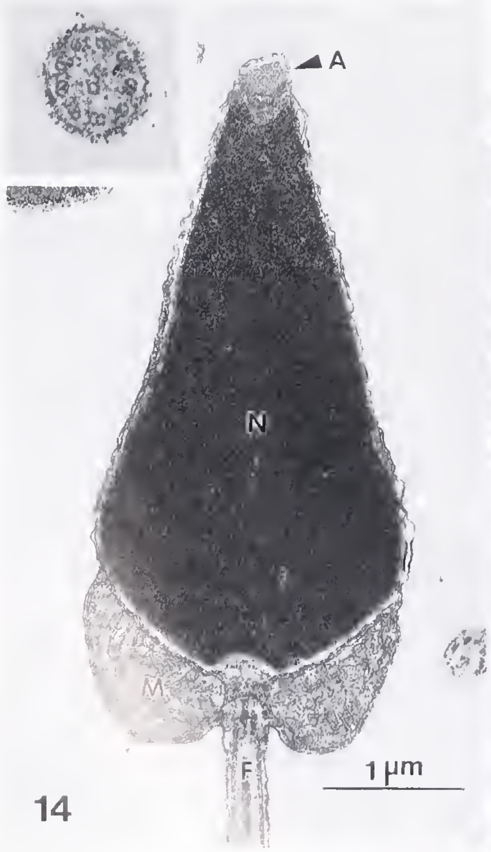

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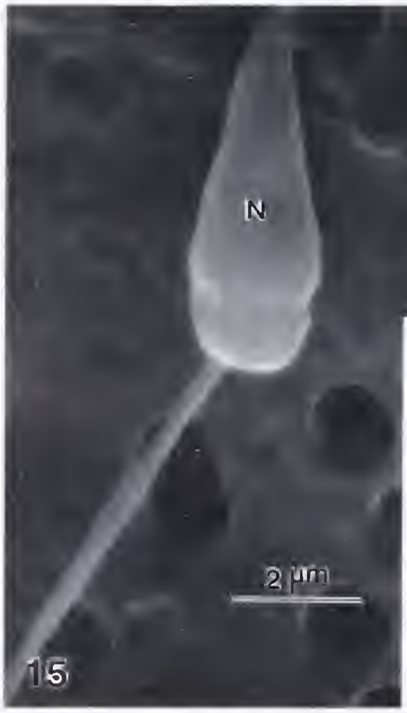

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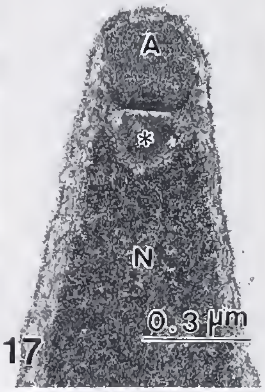

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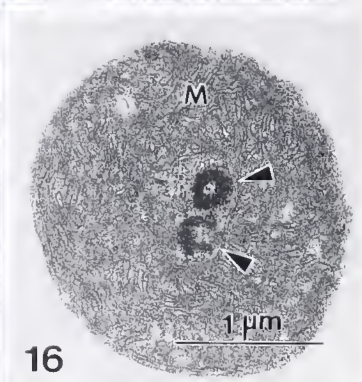

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The primary flagellum projects from the spermatid near the intercellular bridge (Fig. 18) while a secondary axoneme remains intracellular (Figs. 19, 20). The proximal centriole bears a fibrous appendage that extends into the shallow centriolar fossa, similar to that described in the euspermatozoa (Figs. 19, 21, 22).

The spermatid nucleus begins a process of nuclear reduction beginning with the formation of a redundant nuclear envelope that divides the nucleus into two closely apposed, approximately equal-sized halves (Fig. 23). The mitochondria congregate at the base of one nucleus (Fig. 23) and both nuclei begin a process of chromatin condensation (Fig. 24). A small proacrosomal vesicle appears near the future apical region of the cell (Fig. 24, insert). The process of chromatin condensation continues in only one nucleus and a shallow centriolar fossa forms at its base (Fig. 25). The second nucleus gradually condenses into an electron-dense inclusion that is cast off as superfluous chromatin (Figs. 26, 27, 31, 36).

The remaining spermatid nucleus elongates and tapers anteriorly. Many nuclei contain a number of nuclear vacuoles (Fig. 30). The proximal centriole and its associated axoneme extends anteriorly and lateral to the nucleus (Fig. 28) with the centriole eventually coming to rest within the centriolar fossa at the base of the nucleus (Fig. 30). Microtubular components of the axoneme formed by the distal centriole separate, with a portion circling the nucleus and the remainder contributing to the posterior flagellum (Figs. 26, 27, 29). The anteriorly directed component of the axoneme extends well beyond the nucleus (Fig. 31), reaching a length of about 75 μm. The fibrous appendage associated with the proximal centriole disappears at this stage.

The mature paraspermatozoon bears an apical acrosome similar to that of the euspermatozoon (Figs. 31–33). A single primary flagellum lies parallel to the long axis of the nucleus and extends about equidistant both anteriorly and posteriorly to create a bipolar-tailed spermatozoon (Figs. 35, 40, 41). This primary flagellum appears to originate from the distal centriole but is joined just anterior to the acrosome by a secondary axoneme originating from the proximal centriole at the base of the nucleus (Fig. 33). Therefore, two 9 + 2 axonemes originating from separate centrioles lie on opposite sides of the nucleus (Fig. 39) and fuse into a single flagellum anteriorly (Fig. 33 and insert). Longitudinal and transverse sections of the distal region of this flagellum show that it is variable in thickness and is composed of disorganized microtubular doublets that taper near the terminal region (Figs. 34, 35, insert). The distal and proximal centrioles lie nearly parallel to each other within the centriolar fossa, surrounded by a single circular mitochondrion (Figs. 37, 38). Microtubules associated with the distal centriole fuse with the primary flagellum soon after emerging from the posterior region of the cell (Figs. 35–37). Scanning electron micrographs of the mature paraspermatozoa show the bipolar-tailed cell and the lateral displacement of the posterior-directed flagellum which creates a rotational asymmetry (Figs. 40–41). The anterior and posterior portions of the flagellum each extend about 75 μm from the nucleus.

Discussion

Sperm dimorphism has not been reported previously in echinoderms although it exists in a number of other invertebrate groups (see reviews by Fain-Maurel, 1966; Roosen-Runge, 1973, 1977; Healy and Jamieson, 1981; Buckland-Nicks *et al.*, 1982; Jamieson, 1987). The possible functions of paraspermatozoa have been discussed by several authors (Nishiwaki, 1964; Fain-Maurel, 1966; Baccetti and Afzelius, 1976; Healy and Jamieson, 1981) and include the transporting of euspermatozoa from one animal to another (see Nishiwaki, 1964), the prevention of premature euspermatozoan dispersal by entanglement in the multiple tails of the paraspermatozoa (Woodard, 1940), and the nutrition and/or stimulation of euspermatozoa by paraspermatozoan breakdown products (see Buckland-Nicks and Chia, 1977). Sperm polymorphism is believed to be under genetic control with environmental factors (Ansley, 1958; Fain-Maurel, 1966) and hormonal control (Buckland-Nicks *et al.*, 1982) playing a secondary role.

Figure 11. Spermatid with granular chromatin in nucleus (N), electron-dense layer present on inner nuclear envelope (arrows) where centriolar fossa is forming. Note appendage (*) projecting from proximal centriole (PC) into centriolar depression. DC, distal centriole; F, flagellum; M, mitochondrion.

Figure 12. Spermatid with elongating nucleus (N) and single mitochondrion (M) surrounding distal (DC) and proximal (PC) centrioles. Note appendage projecting from proximal centriole (*). F, flagellum.

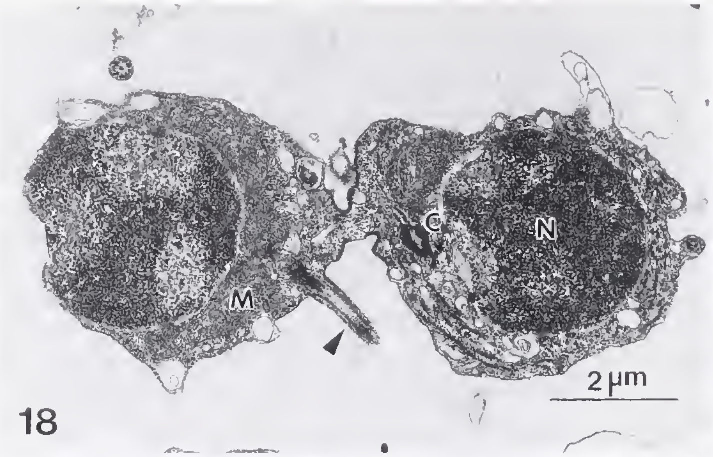
Figure 13. Proacrosomal granule (*) and differentiating subacrosomal fossa (arrows) closely associated with spermatid nucleus (N).

Figure 14. Mature euspermatozoon. A, acrosome; N, nucleus; M, mitochondrion; F, flagellum. Insert: cross section through flagellum showing 9 + 2 pattern.

Figure 15. Scanning electron micrograph of euspermatozoon.

Figure 16. Cross section through middlepiece showing circular mitochondrion (M) and adjacent centrioles (arrows).

Figure 17. Acrosome (A), subacrosomal fossa (*) and nucleus (N) of mature euspermatozoon.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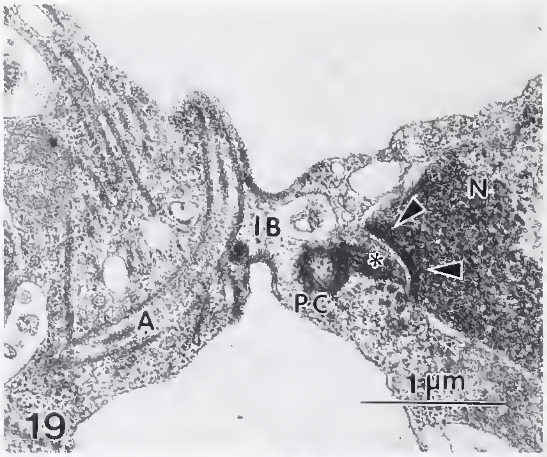
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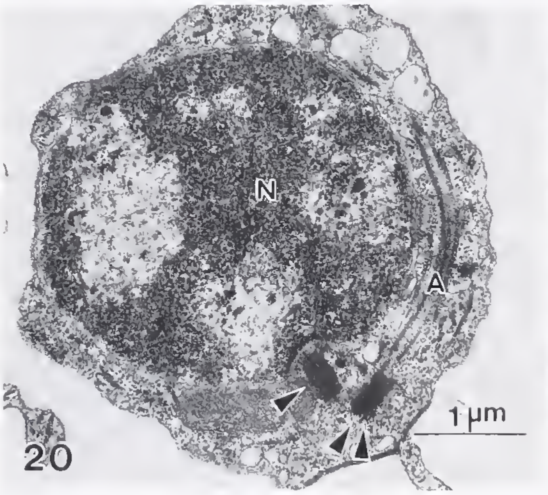
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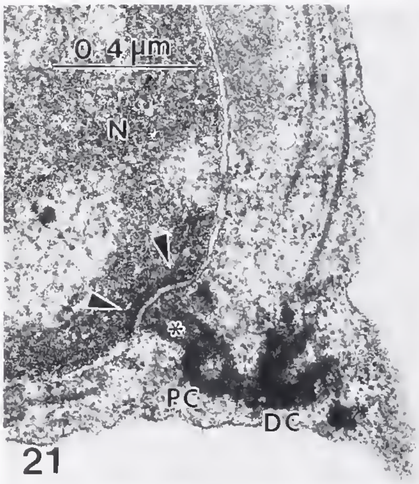
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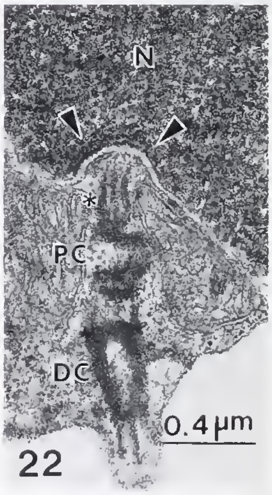
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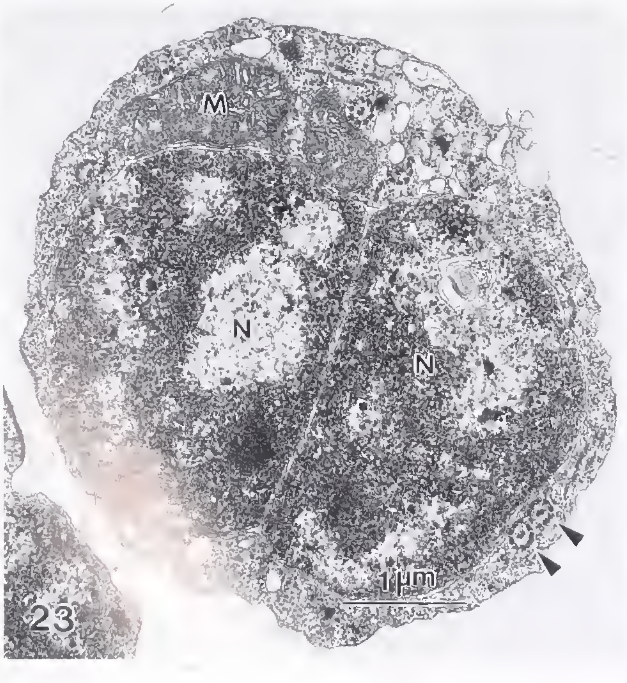
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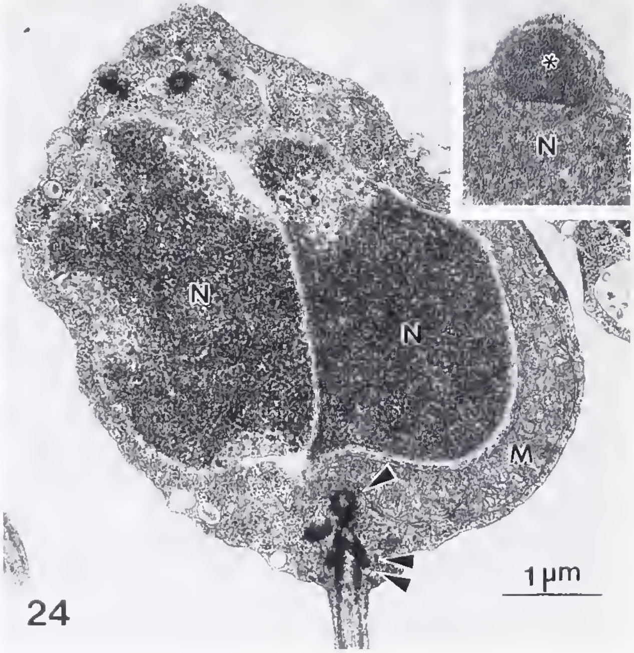
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We were unable to observe spawning in *Phrissocystis multispina* so it is unclear if both the euspermatozoa and paraspermatozoa are actually released under natural conditions. However, we believe it is reasonable to assume that they are, since they were present in approximately equal proportions in the testes of each of the four mature males examined. We do not know the function of the paraspermatozoon because we were unable to perform fertilization experiments. However, the selective pressures responsible for the evolution of sperm dimorphism in this species could relate indirectly to population densities. Abyssal and bathyal echinoderm population densities are generally much lower than those of shallow water echinoderms (Pawson, 1982) and our limited submersible observations of *P. multispina* populations in Hawaii confirmed this generalization. Some mechanism is undoubtedly needed to ensure successful fertilization of eggs in widely dispersed populations. Several mechanisms could facilitate fertilization including aggregation during the breeding season, reproductive synchrony, spermatophore production, copulation, hermaphroditism, self-fertilization, and parthenogenesis. There is no direct evidence that any of these mechanisms are used by *P. multispina.*

Shallow-water echinoids aggregate during spawning seasons (Boolootian, 1966), but it is generally unclear if this results from attraction to conspecifics for purposes of reproduction or from some other cause (Pearse and Arch, 1969). However, pairing for the apparent purpose of spawning has been reported recently (Young, Tyler and Cameron, in prep.) in the deep-sea echinoids, *Stylocidaris lineata* and *Cidaris blakei,* based on *in situ* submersible observations and histological examination of the gonads.

In the absence of spermatophore production or copulation, broadcast spawning of sperm could represent a high-risk fertilization mechanism in *P. multispina* unless there are means to retard sperm dilution or otherwise enhance the probability of fertilization. Pennington (1985) demonstrated with laboratory and field experiments with *Stronglyocentrotus drobachiensis,* that freely spawned sperm are rapidly diluted over short distances resulting in low fertilization rates. Similar results have been obtained for *Diadema antillarum* (Levitan, 1988).

In limited laboratory observations of live and fixed sperm samples from *P. multispina,* we noted that sperm clumping readily occurred due to the entanglement of flagellae. Woodard (1940) conducted experiments with live sperm from the fresh water prosobranch, *Goniobasis,* and convincingly demonstrated that the euspermatozoa became entangled in the multiple tails of the paraspermatozoa, which caused clumping and prevented their wide dispersal. A similar function could be attributed to the bipolar-tailed paraspermatozoa of *P. multispina* as a means of reducing euspermatozoan diffusion during a spawning event.

The dimorphic sperm of *Phrissocystis multispina* are different from those reported in many invertebrates in that both types have acrosomes and similar nuclear morphology. In other species, it is common for the atypical spermatozoon to have greater or lesser amounts of nuclear chromatin or no nucleus at all (Healy and Jamieson, 1981) although in some instances both sperm types appear to have the full haploid chromosome compliment (Jamieson, 1987). Many authors conclude that parasperm are non-fertilizing and therefore do not contribute to the zygote genome. This is undoubtedly the case in those sperm lacking acrosomes, but little is known about the behavior of parasperm in the presence of eggs in any species (Healy and Jamieson, 1981).

The paraspermatozoa of some molluscs have no acrosomes (Yasuzumi *et al.,* 1967) and in others, the acrosomal-like structure observed in these sperm have not been proven to be of Golgi origin (Healy and Jamieson, 1981). Although we did not observe Golgi activity during the development of either sperm type in *P. multispina,* we

Figure 18. Interconnected spermatids of paraspermatozoon. N, nucleus; C, centriole; M, mitochondrion; Arrow indicates flagellum.

Figure 19. Intercellular bridge (IB) connecting two spermatids. Note early formation of centriolar fossa (arrows), fibrous appendage extending from the proximal centriole (PC), and intracellular axoneme (A). N, nucleus.

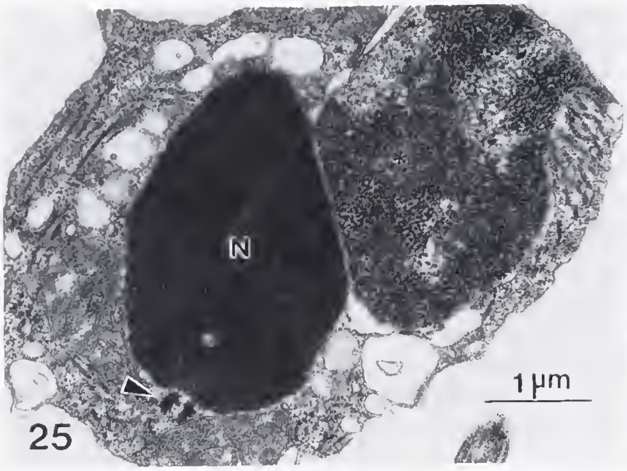
Figure 20. Early spermatid containing proximal centriole (double arrows) with associated intracellular axoneme (A), distal centriole (single arrow) and nucleus (N).

Figure 21. Proximal (PC) and distal (DC) centrioles showing fibrous appendage (*) extending from proximal centriole into centriolar fossa (arrows). N, nucleus.

Figure 22. Fibrous appendage (*) projecting into centriolar fossa (arrows) from proximal centriole (PC). DC, distal centriole; N, nucleus.

Figure 23. Spermatid with two nuclei (N). M, mitochondrion. Arrows indicate cross sections through intracellular axoneme.

Figure 24. Two nuclei with more highly condensed chromatin (N). Proximal (single arrow) and distal (double arrows) centrioles are located at base of cell. M, mitochondrion. Insert: Proacrosomal granule (*) adjacent to nucleus (N).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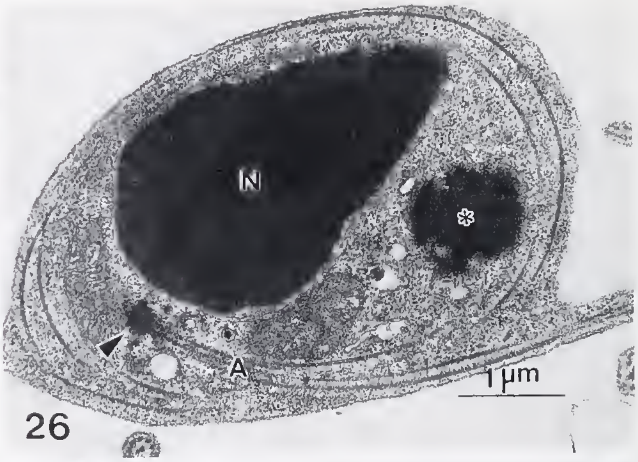
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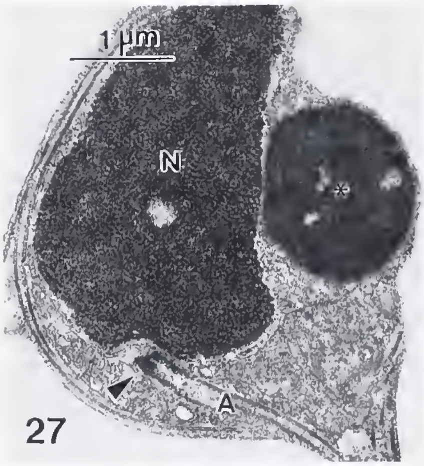
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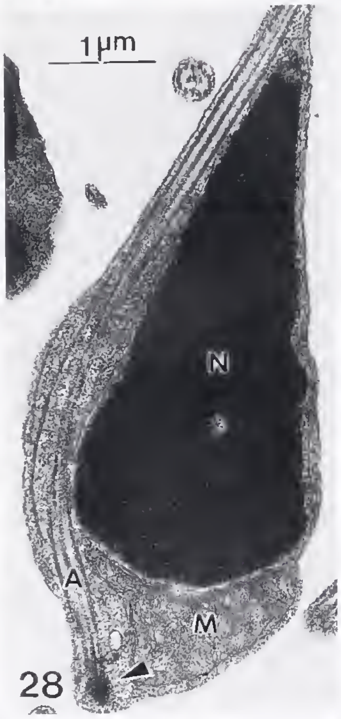
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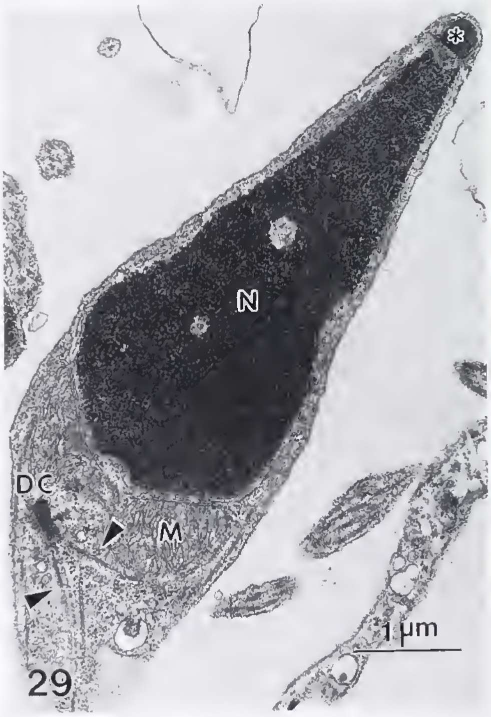
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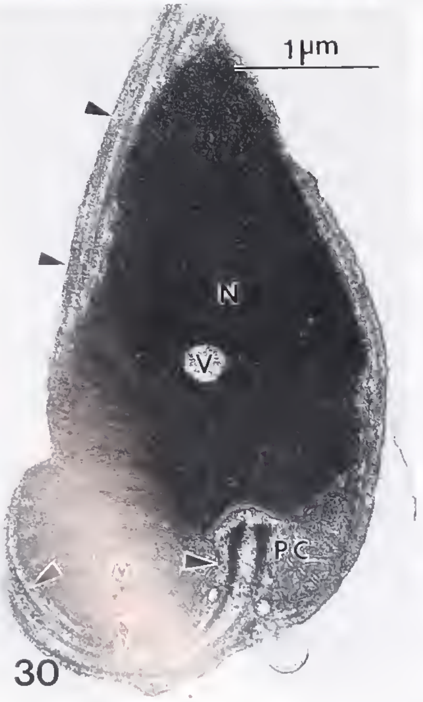
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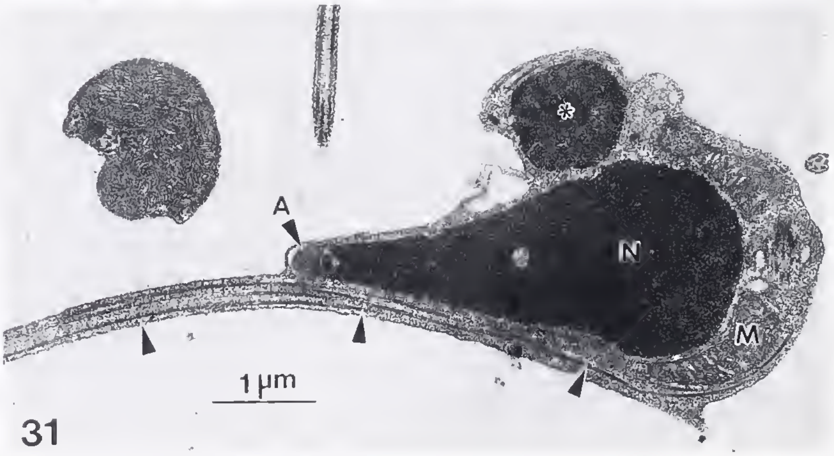
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have chosen to use the term "acrosome" because this structure is morphologically consistent with the acrosomes described in detail in the sperm of numerous echinoid species (Chia and Bickell, 1983). To our knowledge, acrosomogenesis in all echinoderms is associated with Golgi complex activity, so the origin of the acrosome in *P. multispina* sperm is problematical. Most, but not all, metazoan sperm possess acrosomes and they are typically derived from the Golgi complex (Baccetti and Afzelius, 1976). In rare instances, acrosome-like structures are formed without the apparent involvement of the Golgi complex as, for instance, in some gastropod sperm (Garreau de Loubresse, 1971; Eckelbarger and Eyster, 1981). Careful serial sectioning of sperm stages would resolve this question but, until then we will assume that the acrosome observed in the sperm of *P. multispina* sperm has a Golgi origin.

Bipolar-tailed animal sperm are rare, having been reported only from the paraspermatozoa of some archaeogastropod molluscs of the family Neritidae (Nishiwashi, 1964; Tochimoto, 1967). These were light microscopical observations so ultrastructural comparisons to the sperm of *P. multispina* cannot be made. The existence of a bipolar-tailed spermatozoon in *Phrissocystis multispina* is notable because echinoderm sperm are among the best known among invertebrates and are characteristically conservative in morphology (Field, 1895; Tyler and Tyler, 1966; Chia *et al.,* 1975; Eckelbarger *et al.,* in press). Sperm morphology has been described for nearly 130 echinoderm species from shallow water, bathyal and deep sea habitats (Eckelbarger *et al.,* in press). The majority of echinoderms release their gametes directly into seawater and their sperm are largely categorized as "primitive" using the definition of Franzen (1956, 1970). Asteroid and ophiuroid sperm are typically spherical, as are those of crinoids with two known exceptions, the tabloid-headed sperm of *Isometra vivipara* (Holland, 1976a), and the conical-headed sperm of *Antedon bifida* (Holland, 1976b). Echinoid sperm heads are usually conical while holothuroid sperm heads are spherical with two known exceptions, the cylindrical-headed sperm of *Cucumaria lubrica* (Atwood and Chia, 1974) and the tabloid-headed sperm of *Cucumaria pseudocurata* (Atwood, 1975). Recently, however, an unusual spermatozoon was described from the bathyal echinoid, *Aspidodiadema jacobyi,* in which the head contains a thin, tapering, sickle-shaped nucleus reaching 27 μm in length (Eckelbarger *et al.,* in press).

Recent ultrastructural studies of sperm were reported from museum specimens of *Xyloplax turnerae,* a deep-water representative of the newly erected class of echinoderms, the Concentricylcoidea (Healy *et al.,* 1988). The sperm are highly aberrant relative to other echinoderms, consisting of an elongate, internally segmented acrosome; a long tapering nucleus; a single flagellum attached via a centriolar rootlet to the anterior portion of the nucleus; and a single elongate mitochondrion located posterior to the nucleus. The authors concluded that the numerous specialized features of this sperm suggest substantial genetic differences between the Concentricycloidea and other echinoderms and offers strong support for the recognition of the new class proposed by Baker *et al.* (1986) and Rowe *et al.* (1988). The authors dismissed any obligate correlation between modified sperm morphology and abyssal existence because a parallel ultrastructural study of the abyssal asteroid *Caymanostella* sp. from the same habitat showed it to be typical of other asteroid sperm.

However, several recent findings suggest that deep-sea echinoderm populations have undergone a series of novel reproductive adaptations in response to selective pressures apparently unique to deep-sea habitats. A structurally new type of ovary has been described from the bathyal holothuroid *Holothuria occidentalis,* in which podocytes, cells usually involved in fluid ultrafiltration in excretory organs, are intimately associated with developing oocytes (Eckelbarger *et al.,* 1988). Furthermore, an examination of the sperm from 23 species of bathyal echinoderms revealed a significant positive correlation between head length (acrosome, nucleus, and

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Figure 25. Two nuclei within a spermatid showing the highly condensed primary nucleus (N) and the degenerating secondary nucleus (*). Arrow indicates centriole.

Figure 26. Spermatid containing elongating nucleus (N), degenerating nucleus (*) and distal centriole (arrow) with associated axoneme (A).

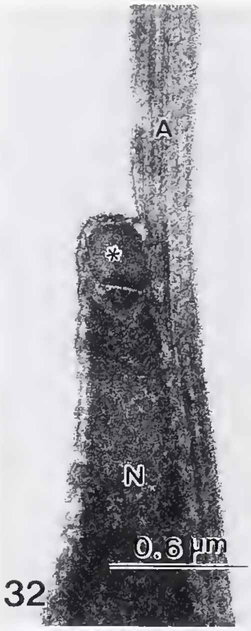
Figure 27. Distal centriole (arrow) and associated axoneme (A) in spermatid. N, nucleus; *, degenerating nucleus.

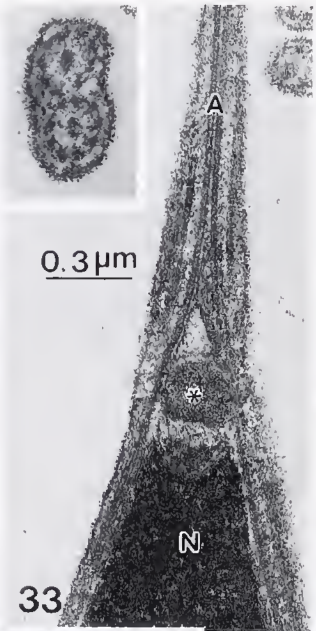
Figure 28. Distal centriole (arrow) and associated axoneme (A) lying adjacent to long axis of nucleus (N). M, mitochondrion.

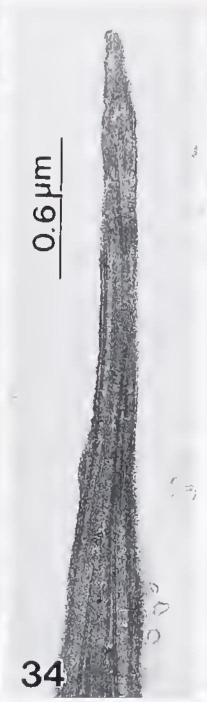
Figure 29. Distal centriole (DC) with splayed axoneme (arrows). M, mitochondrion; N, nucleus; *, acrosome.

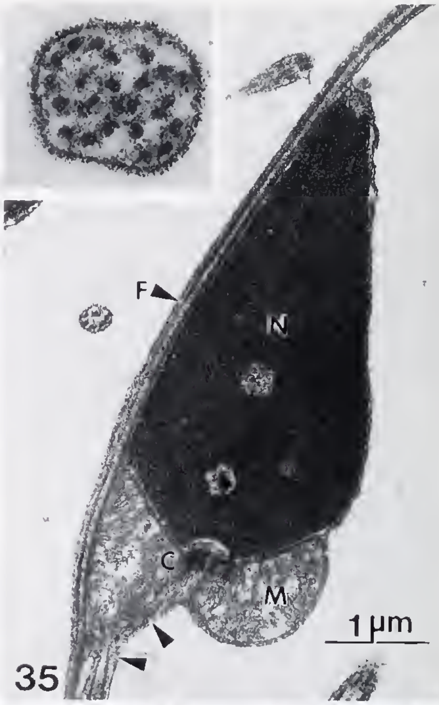
Figure 30. Proximal centriole (PC) and anteriorly directed axoneme (arrows). M, mitochondrion; N, nucleus.

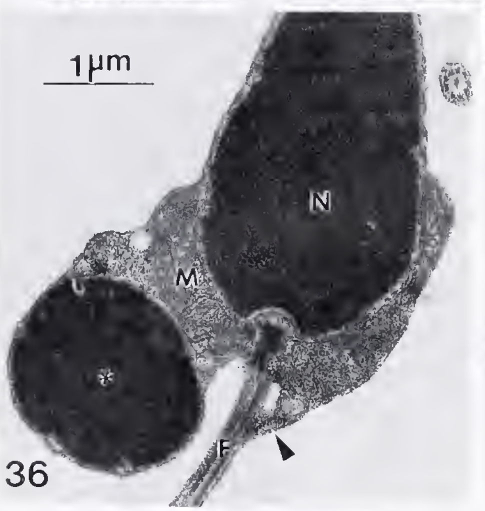
Figure 31. Advanced spermatid bearing anteriorly directed portion of primary axoneme (arrows), terminal acrosome (A) and degenerating nucleus (*). M, mitochondrion; N, nucleus.

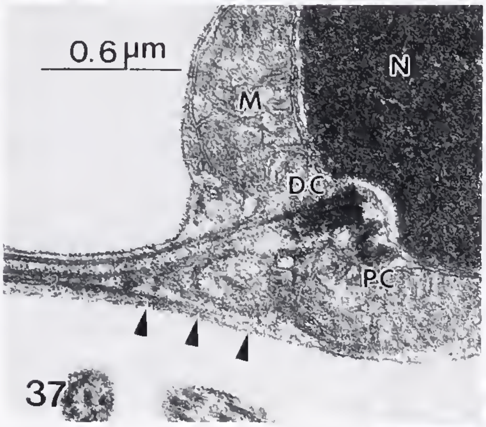











0.6 μm
N
M
DC
PC
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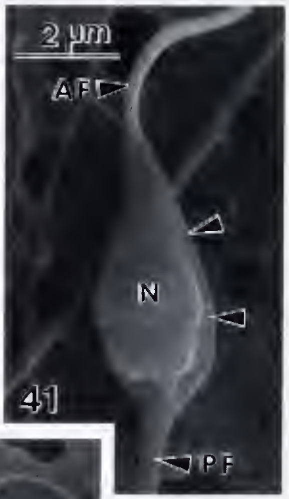
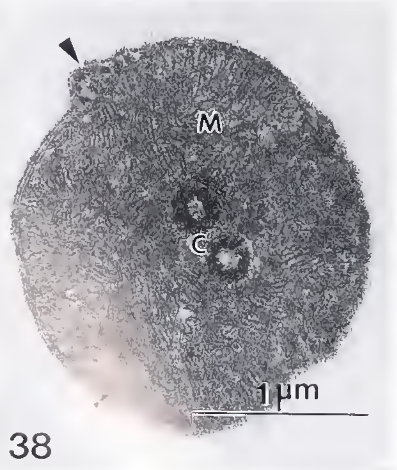
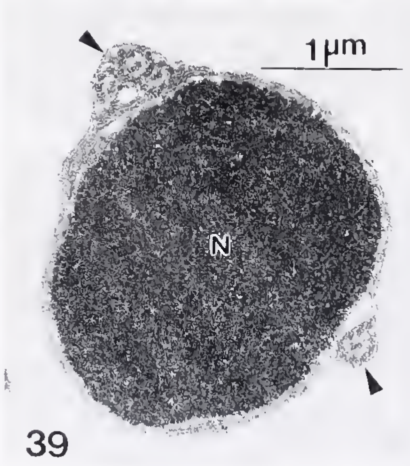

2 μm
AF
N
PF
41





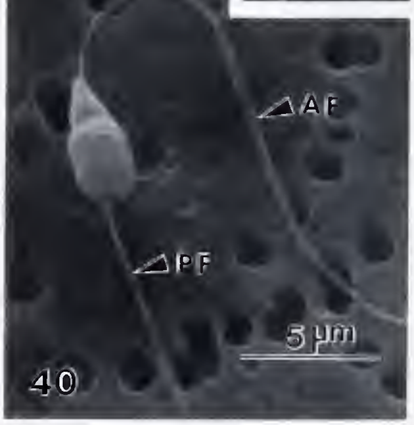


Figure 32. Apical region of paraspermatozoon nucleus (N) showing acrosome (*) and anteriorly directed axoneme (A).

Figure 33. Apical region of nucleus (N) showing acrosome (*) and lateral components of two lateral

middlepiece) and depth distribution among echinoids. Included in these observations is the aberrant sperm from the bathyal echinoid *Aspidodiadema jacobyi* (Eckelbarger *et al.*, in press). Finally, a unique type of larval development was described in *A. jacobyi* in which small (90 μm) eggs spend sequential portions of their planktonic development in lecithotrophic and planktotrophic phases (Young *et al.*, in prep.).

The unusual morphological specializations reported in deep-water echinoderm sperm can largely be attributed to alterations in fertilization biology. Franzen (1956) noted that structural modifications of sperm often are associated with altered demands on the mobility of the sperm, particularly when they must swim through a viscous medium. The evolution of derived sperm reported in the externally brooding holothuroids *Cucumaria pseudocurata* (Atwood, 1975) and *C. lubrica* (Atwood, 1974; Atwood and Chia, 1974) has been attributed to the presence of viscous mucus surrounding the egg mass through which the sperm must pass (Atwood and Chia, 1974; Atwood, 1975). In *Aspidodiadema jacobyi*, eggs are released in a mucus mass through which presumably the modified sperm must pass (Eckelbarger *et al.*, in press).

In the Concentricycloids *Xyloplax turnerae* and *X. medusiformis*, it is believed that sperm transfer involves copulation between male and female using modified spinelets of the male (Rowe *et al.*, 1988). The existence of highly aberrant spermatozoa in these species correlates nicely with the mechanism of fertilization because modification of sperm morphology is directly related to the evolution of internal fertilization (Franzen, 1956, 1966; Afzelius, 1977). Egg size may also influence the length of the sperm head in some species. An analysis of sperm head length and egg size in bathyal populations of echinoids and holothuroids revealed a strong positive correlation indicating that larger eggs are fertilized by larger sperm (Eckelbarger *et al.*, in press). Franzen (1983) noted a similar correlation between the evolution of elongated sperm nuclei and large yolk-rich eggs in bivalve molluscs. However, one must be cautious in drawing correlations between sperm modifications and fertilization biology in echinoderms because in some ophiuroids, asteroids, and holothuroids, primitive sperm are not always encountered in externally fertilizing species and derived sperm are not consistently observed in species in which fertilization is internal (Chia *et al.*, 1975; Janssen, 1984; Healy *et al.*, 1988).

Despite the widespread use of echinoid sperm in modern developmental biology studies, there is a paucity of information regarding their development (Chia and Bickell, 1983). Sperm morphology is known for nearly 70 echinoid species (Chia *et al.*, 1975; Eckelbarger *et al.*, in press), but ultrastructural studies of echinoid spermatogenesis are confined to only four species (Longo and Anderson, 1969; Cruz-Landim and Beig, 1976). Many features of development of the dimorphic sperm of *Phrissocystis multispina* parallel those of other echinoids as well as other echinoderms. However, differentiation of the nucleus and flagellum are notable exceptions.

During early spermiogenesis in the paraspermatozoa of *Phrissocystis multispina*, chromatin reduction is accomplished by the formation of a redundant nuclear envelope in a manner similar to that reported in some ver-

axonemes which fuse into a single axoneme (A) just anterior to the acrosome. Insert: cross section through two 9 + 2 axonemes just anterior to acrosome.

Figure 34. Longitudinal section through tapering terminal region of anteriorly directed flagellum showing tapering tip.

Figure 35. Longitudinal section through paraspermatozoon showing anteriorly and posteriorly directed flagellum (F) and two adjacent centrioles (C). Arrows indicate microtubular components extending from distal centriole which join the posteriorly directed portion of the flagellum. M, mitochondrion. Insert: cross section through terminal region of anteriorly directed axoneme shown in Figure 34. Note disorganized arrangement of microtubules.

Figure 36. Spermatid with superfluous nuclear chromatin inclusion (*). Arrow indicates branching of posterior flagellum (F) as it extends anteriorly alongside the nucleus (N). M, mitochondrion.

Figure 37. Middlepiece region of paraspermatozoon showing the association of the microtubular elements of the primary flagellum (arrows) with those emanating from the distal centriole (DC). PC, proximal centriole; M, mitochondrion; N, nucleus.

Figure 38. Cross section through the middlepiece region showing the circular mitochondrion (M), the parallel centrioles (C) and the oblique section through the laterally positioned axoneme (arrow).

Figure 39. Cross section through the central nuclear region (N) showing axonemes on both sides of the nucleus (arrows).

Figure 40. Scanning electron micrograph of mature paraspermatozoon showing posteriorly (PF) and anteriorly directed (AF) components of the flagellum.

Figure 41. Scanning electron micrograph of nuclear region (N) of paraspermatozoon showing anteriorly (AF) and posteriorly directed (PF) components of flagellum. Arrows indicate primary axoneme lateral to nucleus. Compare this micrograph to TEM section shown in Figure 35.

tebrate sperm (see Yasuzumi, 1974). This results in a division of the nucleus into two approximately equal regions, one of which degenerates and is apparently cast off. During development of the apyrene carrier spermatozoon in the mollusc *Fusitriton oregonensis*, the nuclear envelope pinches off a series of lobes from the nucleus, resulting in discrete vacuoles (Buckland-Nicks *et al.*, 1982) termed "carymerites" by earlier authors (Goldschmidt, 1916). In *F. oregonensis*, the caryomerites undergo intracellular digestion while in the "atypical" sperm of other animals, nuclear remnants are excreted from the cell or converted into glycoproteins and stored in the middlepiece (see Buckland-Nicks *et al.*, 1982, for discussion).

The details of the process are not entirely clear, but the primary flagellum in the paraspermatozoon of *Phrissocystis multispina* appears to be formed by the distal centriole as in other echinoderm sperm. However, the proximal centriole, which plays no role in flagellum formation in other echinoderm sperm (Chia and Bickell, 1983), forms an intracellular, secondary axoneme which fuses with the primary flagellum. The function of this secondary axoneme is unknown, but we assume it is immotile and perhaps provides structural support to the primary flagellum during propulsion.

Acknowledgments

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The Annual Pattern of Feeding, Growth, and Sexual Reproduction in *Cyanea* (Cnidaria: Scyphozoa) in the Niantic River Estuary, Connecticut

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Abstract. The seasonal occurrence, population dynamics, feeding, growth, reproduction, and timing of planular deposition was determined for a semelparous annual population of *Cyanea* medusae in the Niantic River estuary, Connecticut, which was sampled for 14 years. The annual pattern was highly predictable. Their ephyrae appear shortly after ice-out in late February, medusae with gonads occur in late April–early May, and are reproductive (bearing planulae on their oral folds) by late May–early June. At this time (early to mid-June), the medusae deteriorate, disappearing from the plankton in late June–early July. Population size, similarly, exhibits small variation, showing only a 5-fold difference in abundance. With deterioration, the frequency of feeding medusae declines; otherwise, they are opportunistic predators on seasonally numerous zooplankton, including fish. Growth shows two phases: first, a three month period of rapid exponential growth when medusae mature; and second, a one and one half month period of decline in average size of individuals as the larger medusae die. Medusae exhibit a sequence of "organ" loss prior to death: first, the tentacles, then the oral folds, and last, the gonads. Coincident with the disappearance of these organ-deficient medusae from the water column, their planulae first appear attached to shells in the benthos. The deterioration of medusae, and in particular the loss of planulae-bearing oral folds, may be requisite for the mass release of their planulae.

Introduction

The general biology, ecology and life history of the Scyphozoa are well-known, (*e.g.*, Agassiz, 1862; Mayer, 1910; Thiel, 1959, 1962; Russell, 1970). Jellyfish, such as *Cyanea*, are found in coastal seas worldwide. They possess a benthic polyp stage (larva) which reproduces asexually to give rise to ephyrae; these grow into dioecious, planktonic medusae (adults) that produce planulae (motile stereogastrulae), and the latter settle to the benthos to form polyps, completing the cycle. Studies of the asexual reproduction by polyps both in the laboratory (*e.g.*, Custance, 1964; Spangenberg, 1965; Sugiura, 1965; Kakinuma, 1975; Cargo and Rabenold, 1980; Calder, 1982) and in the field (*e.g.*, Thiel, 1962; Gröndahl and Hernroth, 1987; Gröndahl, 1988; Brewer, unpubl.) show the effect of food and seasonal temperature on this process. On the other hand, most past studies of scyphozoan medusae have been taxonomic and zoogeographical (*e.g.*, Mayer, 1910; Kramp, 1961), or have focused on their local seasonal and spatial distributions (see below), and the details of their sexual reproduction and ecology remain obscure.

The awareness that scyphozoan medusae may have a major impact on the dynamics of plankton communities (*e.g.*, Phillips, *et al.*, 1969; Shushkina and Musayeva, 1983; Feigenbaum and Kelly, 1984) and especially on fish populations (*e.g.*, Möller, 1979, 1980a, b, 1984; van der Veer, 1985) has stimulated a renewed interest in jellyfish; that they, and other gelatinous plankton, eat fish is established (reviewed in Purcell, 1985). Larson (1986a) examined growth and production rates of gelatinous plankton in pelagic food webs; Shenker (1985), Larson (1986b), and Schneider (1988) provide data on the chemical composition of medusae necessary for such studies. Fancett (1988) showed that *Cyanea* is a selective predator on fish, and an evaluation of their influence on the abundance of both ichthyoplankton and zooplankton is given by Fancett and Jenkins (1988). Larson

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(1987a) describes the predatory impact of hydromedusae and ctenophores on commercially important zooplankton. The effect of different density combinations of hydro- and scyphomedusae and their larval fish prey has been experimentally examined in the field by de Lafontaine and Legget (1988) using *in situ* enclosures.

Field studies of several years duration help to determine the significance of such predator-prey relationships as well as to understand the life history of the predator species concerned. Few long-term field investigations of *Cyanea* have been done. Exceptions are the studies of seasonal abundance by Russell (1931) in the Plymouth area, van der Maaden (1942) and Verwey (1942) along the Dutch coast, and by Rasmussen (1973) in the Isefjord, Denmark; and more recently, in its role as a predator, by NUSCo (1986) in the Niantic River, Connecticut, and by Hernroth and Gröndahl (1983), Gröndahl and Hernroth (1987), and Gröndahl (1988) off the west coast of Sweden. Earlier, Fish (1925) reported the seasonal occurrence of *Cyanea* in the Woods Hole region of Massachusetts for the years 1893–1907, during two years of which they were absent. A difficulty for long-term studies of these medusae is their large year-to-year variation in abundance (*e.g.*, van der Maaden, 1942; Verwey, 1942; Rasmussen, 1973).

This study of *Cyanea* was done on a circumscribed estuarine population in the Niantic River, Connecticut; here, medusae occur from early spring to early summer every year (Marshall and Hicks, 1962). I sampled medusae at this site for 14 years to determine the annual timing of sexual maturation (appearance of gonads) and of reproduction (presence of blastulae and/or planulae on the oral folds); the extent to which population size varies from year to year; their diet, feeding frequency, and seasonal food preferences; the average growth rate of medusae; and when planulae are released by medusae which marks the beginning of the asexual benthic phase of their life cycle.

Materials and Methods

The site of this study was the estuary of the Niantic River, Waterford, Connecticut, described in Marshall (1960), Marshall and Wheeler (1965), and in McGill (1972). It is a typical coastal estuary except that its mouth into Long Island Sound (Niantic Bay) is restricted to a narrow channel by a railroad embankment. Sampling for medusae, and recording of surface temperature, was done between February and July, 1973–1986, at weekly or fortnightly intervals.

Medusae were collected from docks at Smith Cove (on the western margin of the Niantic River) using a dip net, and on each sample date a relative measure of abundance of medusae was obtained by counting the number seen during a 30-min period (1981–1986). In the laboratory, their size, state of reproduction, condition (intact or deteriorating), and the contents of their gastrovascular cavity, was determined. The maximum diameter of each medusa (3220 individuals) was measured to the nearest 1.0 mm by lying specimens on their aboral surface in a dish of only enough seawater to flatten them. To determine the relationship between diameter and wet weight, the volume (ml) of 41 medusae, 42 to 215 mm diameter, was determined by liquid displacement and expressed as wet weight (g) (Omori and Ikeda, 1984; McCauley, 1984); the wet weights were used to calculate k, the exponent for exponential growth (Omori and Ikeda, 1984) to obtain the instantaneous growth rate ($k = 1/T\ [\ln W_T - \ln W_0]$, where T is time [days] and W_0 and W_T are the initial wet weight and wet weight at time T, respectively). For each sample, and using up to 50× magnification as necessary, the number of medusae with undeveloped gonads, with testes or ovaries, and with or without blastulae or planulae on the oral folds, was recorded. Food items in 2060 individuals collected in 1980–1986 were identified.

Two methods were used to determine the timing of planula release. First, a plankton net (diameter = 24 cm; mesh = 76 mμ) was used to collect planulae in the water column during the period when medusae were reproducing. Second, sampling for planulae attached to benthic substrate occurred weekly or fortnightly between 7 April and 9 September 1981, to obtain planulocysts (newly settled planulae); these were counted, at 50×, on shells obtained by dredging the channel of the Niantic River (depth at MLW = 4 m) with a 9.1-m otter trawl. On most dates, a 3-gallon bucket of shells was gathered for examination (total = 3039 shells).

Results

Cyanea medusae exhibit four stages of development: immaturity, sexual maturation, reproduction (presence of blastulae), and deterioration as shown in Figure 1 for the years 1973–1986. Maturation occurs 43 days (range, 28–58 days) after the initial presence of medusae; gonads usually appear during the latter half of April. From then through the first half of May, fertilization and ensuing embryogenesis brings development to the blastula stage after an additional 17 (10–24) days; planulae (not shown in Fig. 1) appear on oral folds of female medusae an average of 11 (5–15) days later. Intact medusae brooding blastulae and planulae are present for 18 (11–25) days. Deteriorating medusae, which bear planulae until they lose their oral folds, occur for another 25 (11–39) days. Deterioration of medusae occurs in the same sequence each year: first, the tentacles disintegrate; next, the oral folds are lost; this is followed by deterioration of the gonads; and finally, after the necrosis of the rest of the sub-

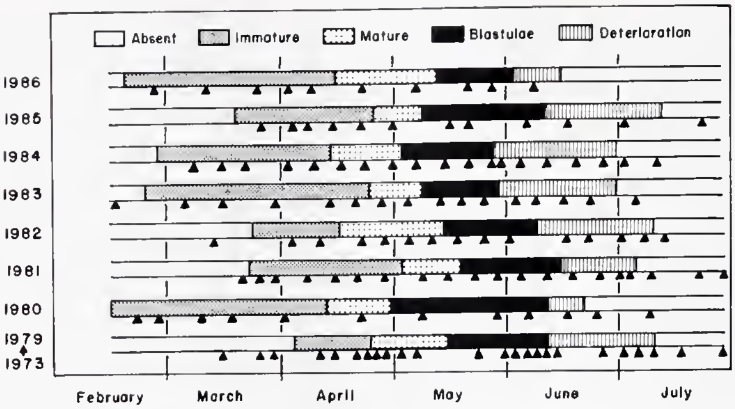

Figure 1. The seasonal occurrence and characteristics of *Cyanea* medusae in the Niantic River estuary, 1973–1986. Solid triangles indicate sample dates. Sampling was less frequent, 1973–1979, and the pattern shown represents the average for those years.

and exumbrellar epithelium, only the mesoglea remains. Even in an early deteriorated state, the medusae cannot feed (see below). Medusae, in one or another stage, occur for about 4 months each year before they disappear. The average last date to find deteriorated medusae in the plankton is 29 June.

The small annual variation in the occurrence of the different medusa stages is paralleled by the nearly constant population size of *Cyanea.* The comparison of their peak annual abundance (Fig. 2) shows that the maximum abundance of medusae (always occurring between late April and mid-May) alternates between relatively large populations in even years (1982, 1984, 1986) and relatively low populations in the odd years (1981, 1983, 1985). However, the largest population (recorded in 1986) was only 4.2 times that of the smallest (1985).

The content of their gastrovascular cavity (Fig. 3) is as predictable as is the temporal occurrence of their different stages and the range in their population size. From March through the first half of May, an average of at least 65% of medusae have copepods in their gastrovascular cavity; during the first half of this period many also contain *Palaemonetes* and *Mysis,* replaced by amphipods (mostly gammarids, but a few caprellids) during the second portion of this period. In the latter half of May, when many medusae also contain *Nereis* in their gastrovascular cavity, the percentage that are feeding is halved (from 85% to 41%); this coincides with the deterioration of medusae. This decline in proportion of feeding medusae continues, until in July it is usual to find the gastrovascular cavity empty. Most of the medusae which are still feeding late in the season contain veliger larvae. Less than 2% of medusae contain 12 other species of zooplankton (Cnidaria [ephyrae, *Sarsia, Nemopsis, Rathkea*]; nemertean larvae; Rotifera; polychaete larvae; crustacean larvae [nauplii, megalops, cypris]; Isopoda; and Ostracoda). On the other hand, a consistent proportion (18.9 ± 7.6%) contain fish eggs, yolk-sac larvae, fry, or adults (*Ammodytes, Fundulus, Gasterosteus, Syngnathus*). The percentage of these medusae containing fish eggs was 29%; yolk-sac larvae, 35%; fry, 18.7%; and adults, 17.4%.

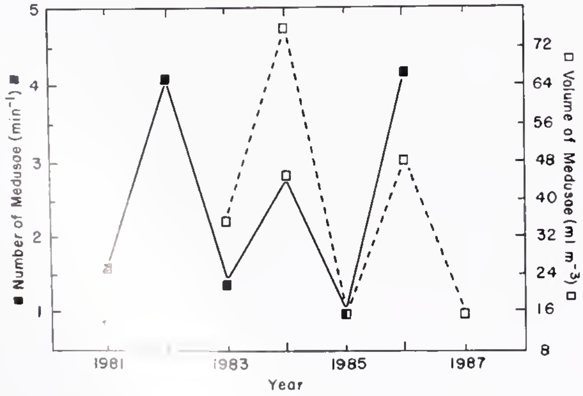

Figure 2. The relative number (this study) and volume (NUSCo, 1988) of *Cyanea* medusae in the Niantic River estuary, 1981–1987. (See Discussion for NUSCo data.)

The growth of medusae follows the same pattern each year (Fig. 4). From their first appearance until the second half of May their growth is exponential. During this time, the fastest growth was 5.6 mm diameter week^{-1}; the slowest was 2.8 mm week^{-1}; and the overall average was 5.0 mm week^{-1}. Wet weight, determined from the relationship shown in Figure 5, was used to calculate the instantaneous daily growth rate (k): k = 0.13, 0.02, and 0.06 for the fastest and slowest growth, and the overall average, respectively. The period of exponential increase ends in June, when the average size of medusae declines

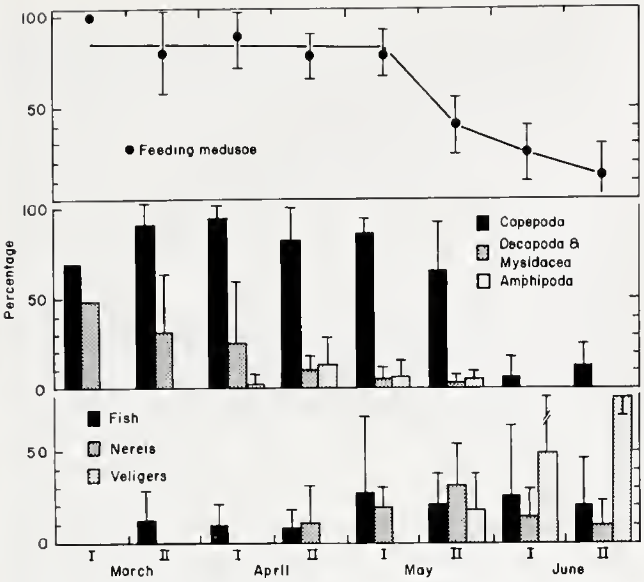

Figure 3. The percentage (±S.D.) of feeding medusae (1980–1986) in the Niantic River estuary, and the average percentage (±S.D.) which contained the indicated taxon in their gastrovascular cavity in the half-month shown.

due to death of larger individuals (Fig. 6). The overall average temperature (and range) at which deterioration occurs is 19.1 ± 2.3°C (16.7°C to 23.3°C).

The onset of reproduction (females with blastulae) is also shown in Figure 6. This occurs earliest in the largest medusae, but even the smallest medusae mature and reproduce before all individuals eventually disappear (between 7 July and 13 July in 1982). The average size (all years) of the earliest 10% of medusae which reproduce is 124 ± 16 mm, and that for the latest 10% of reproducing medusae is 39 ± 7 mm; but the average age of the smaller

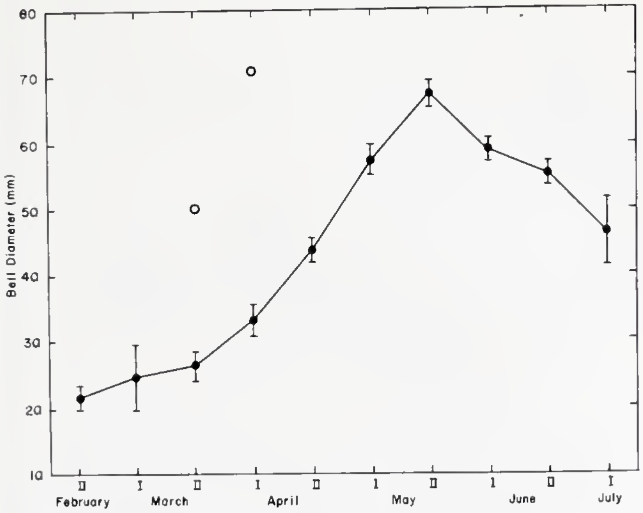

Figure 4. The growth (mean ± 0.95 c.l.) of *Cyanea* medusae in the Niantic River estuary, by half-month interval, from 1973 to 1986. Medusae in 1985 (open circles) are not included in the calculations.

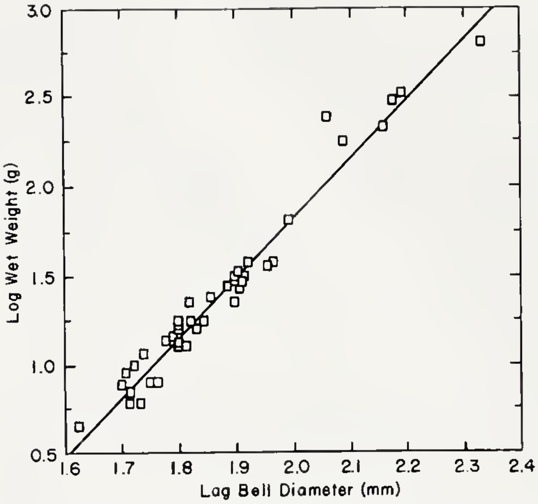

Figure 5. Log-log plot of the relationship between wet weight and bell diameter for *Cyanea:* log wet weight (g) = 3.34 log bell diameter (mm) − 4.85 (R^2 = 0.964, F = 1047, P = 0.0001).

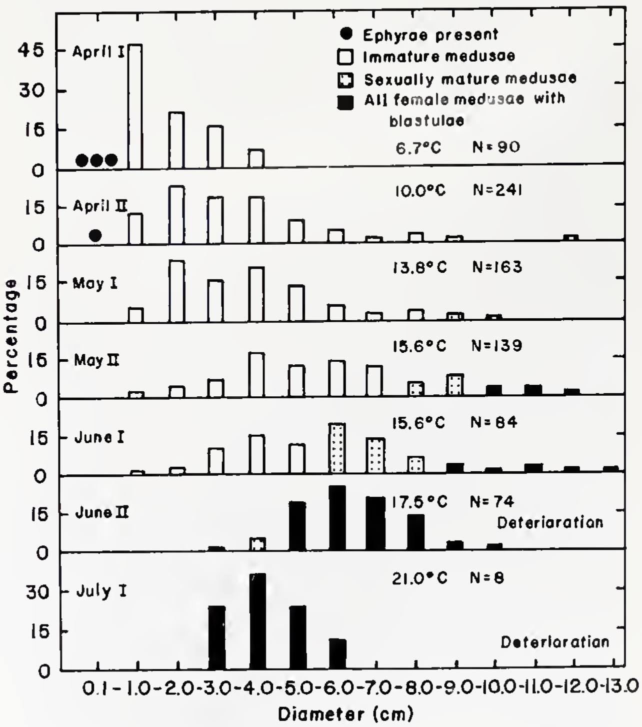

Figure 6. The size frequency distribution for *Cyanea* medusae collected in 1982. The frequency of individuals in each size class from samples in each half of a month are combined. The state of maturity, reproduction (females with blastulae), and condition are indicated. The temperature shown is the average for the half-month periods, and N is sample size.

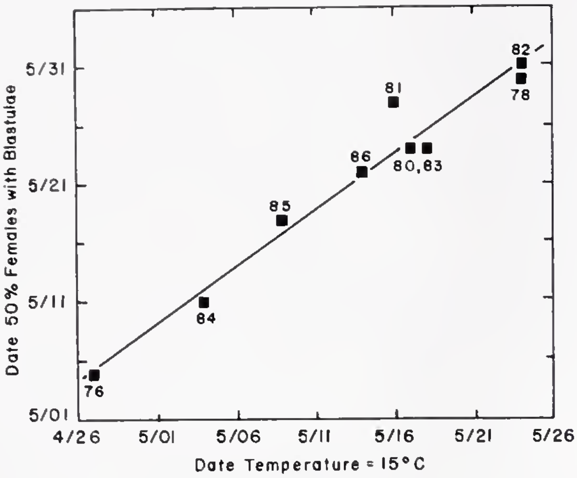

Figure 7. The relationship between temperature and the onset of reproduction (appearance of blastulae on the oral folds) in *Cyanea*. Numbers beside the points indicate the year.

medusae is up to 45.0 ± 10.6 days older than the larger ones. The sex ratio of *Cyanea* is 1:1 (n = 1239).

Sexual development and the onset of reproduction, shown in Figures 1 and 6, is correlated with the increase in temperature during May (and late April). The date that 50% of females bear blastulae on their oral folds is plotted in Figure 7 against the date that the surface water temperature attains 15°C for the nine years that the data permit such calculations ($R^2 = 0.96$, $F = 156$, $P \ll 0.001$). The average female produces blastulae 8 ± 2 days after the surface water temperature reaches 15°C; the blastulae then develop to form planulae.

The attachment of planulae to hard substrate in the benthos occurs predominantly after medusae deteriorate, and particularly after they disappear from the water column (Fig. 8). The proportion of shells bearing attached planulae of *Cyanea* (distinguished from those of other Scyphozoa because they uniquely encyst prior to forming a polyp) increases significantly ($P < 0.001$) at this time over that for the two samples immediately prior to the total disappearance of medusae; the difference among the five later samples is non-significant ($0.10 < P < 0.50$). The number of planulocysts found on shells just before the medusae disappear averages 6 ± 0.7; after the disappearance of medusae, the number averages 168 ± 107. Planulocysts were not observed on six sample dates prior to their first appearance shown in Figure 8, nor were planulae found in the plankton during this period even when a net was passed beneath aggregations of gravid medusae.

Discussion

The population of *Cyanea* medusae in the Niantic River estuary is remarkably stable, both in the annual timing of its growth, maturation, and reproduction, and in its annual abundance from year to year. These data on relative abundance are consistent with those obtained using standard oceanographic methods (Fig. 2). The Northeast Utilities Environmental Laboratory has recorded medusa volume in their plankton tows in the Niantic River since 1983 (NUSCo, 1984), and in three out of the four years that our sampling has overlapped, the regressions comparing our results are highly significant (1983, $R^2 = 0.959$; 1984, $R^2 = 0.962$; 1986, $R^2 = 0.828$); in 1985, the fit was poor ($R^2 = 0.297$). In 1985, the size of medusae did not fit the pattern observed in other years (Fig. 4), and the numbers I obtained then might not correspond to their volume measurements. Their data on volume have the same alternating pattern of peak abundance and show a five-fold difference between the largest population and the smallest (from data in NUSCo, 1988), and is close to what I obtained (a 4-fold difference). The density of medusae, estimated (NUSCo, 1987) by converting volume to numbers from size frequency data, shows a similar range (from 1 to 4 medusae m^{-3}) during the years 1983–1984.

There are few other long-term studies of scyphozoan abundance. Rasmussen (1973) reports relative abundance of *C. capillata* in the Isefjord (Denmark) between 1950 and 1962, by indicating the years that were exceptionally low (3 years) or high (5 years); the remaining five years presumably representing an undefined "normal" abundance. van der Maaden (1942) gauges abundance along the coast of Holland, between 1933 and 1937, by counts of stranded individuals. These data show about a 35-fold difference between the lowest and highest numbers for *C. capillata* and *C. lamarcki*, but the extent to which these reflect population size off shore is not clear.

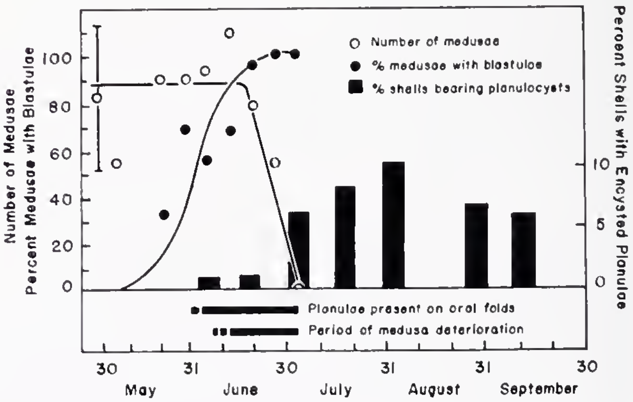

Figure 8. The relative numbers of *Cyanea* medusae in the plankton, the proportion with blastulae on their oral folds, the period when planulae were present and the medusae were deteriorating, and the frequency of attached, encysted planulae on shells during 1981. Number of medusae is number min^{-1} × 50; the average ± S.D. for April is shown.

There is a 13-fold range in relative abundance, *in situ,* for *C. lamarcki* in the Plymouth area during 1925–1930 (Russell, 1931). Jellyfish abundance, *in situ,* is reported by Cargo and Schultz (1967) for *Chrysaora quinquecirrha* in Chesapeake Bay between 1960 and 1966. For this species, there is about a 100-fold difference between the lowest and highest peak population, but for the years 1962–1966, the maximum difference is only 6-fold, and was about the same ten years later (Cargo and Rabenold, 1980). Had the present study been of longer duration, the interannual peak populations might have differed to a greater extent. Nonetheless, these abundances generally indicate that variation in numbers of jellyfish among years is often less than the spatial and temporal range observed within a given season (*e.g.,* Thiel, 1962). The populations above (except for *C. lamarcki*) exhibit peak populations which alternate between low and high relative abundance for periods of at least four consecutive years as in this study, but with occasional two-year runs of similar abundance.

The succession of items appearing in the diet of *Cyanea* indicates that it is an opportunistic predator, and that apparently copepods are particularly important. During the time of maximum growth of *Cyanea,* the peak densities of *Pseudocalanus minutus, Acartia hudsonica* (both in mid-March), and *Temora longicornis* (in mid-April) occur; and the population of *Centropages hamatus* is increasing during this period, peaking in mid-June (NUSCo, 1984). The trophic significance of crustacean zooplankton in the diet of medusae is noted by Fraser (1969), Clifford and Cargo (1978), and Möller (1980, 1984). But the prevalence of copepods in the gastrovascular cavity may give a false impression of the trophic ecology of this medusa. Fancett (1988) shows that *Cyanea* exhibits a negative selection for copepods and a strong positive selection for fish (eggs, yolk-sac larvae). The proportion of medusae containing fish in the present study varied widely in different samples (occasionally reaching 100%), but on all dates, at least some medusae had fed on them. The positive relationship ($P = 0.0004$) between growth of medusae (this study) and the density of winter flounder (*Pseudopleuronectes americanus*) larvae (from NUSCo, 1987) is shown in Figure 9; in addition, NUSCo (1986) found a significant negative correlation between the abundance of *Cyanea* and density of winter flounder larvae from 1983 through 1985. If these indicate cause and effect (both medusae and flounder larvae may be independently influenced, directly or inversely, by other factors) then fish, though less frequently encountered in medusae than copepods, are important in the diet of *Cyanea* in the Niantic River. An intensive study of the trophic relations of this population of *Cyanea* using more rigorous methods (*e.g.,* Larson, 1987b; Fancett, 1988) is required to resolve this question or its predatory status, in general. Others report that ctenophores are important in the diet of scyphozoa (reviewed in Larson, 1978; Larson, 1986c). In the Niantic River estuary, *Pleurobrachia* is present with *Cyanea* early in the season, but only in low numbers, so it is probably not a significant prey item. *Mnemiopsis* often is abundant, but this usually is after *Cyanea* has disappeared late in the season; in the three years when their populations overlapped, the numbers of *Cyanea* were declining and individuals were in a deteriorated condition incapable of efficient feeding.

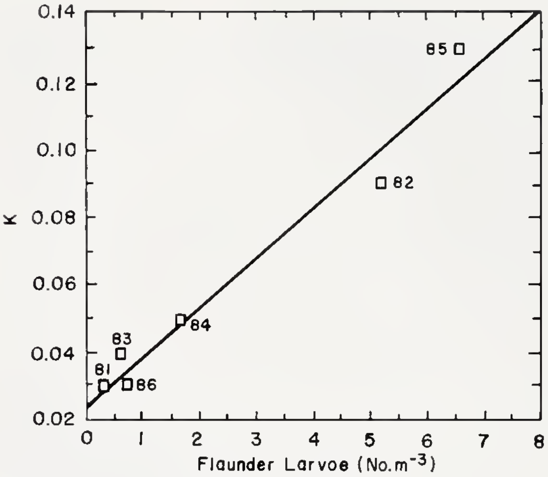

Figure 9. The relationship between instantaneous daily growth rate of *Cyanea,* k, and density of winter flounder larvae in the Niantic River estuary. Numbers beside the points indicate the year.

The average value for daily instantaneous growth rate of the different annual populations of *Cyanea* ($k = 0.06$) is low compared to those reported for other Cnidaria (reviewed in Larson, 1986a), but is the same as that calculated from size frequencies reported in Thiel (1960) for *C. capillata* (0.06). The size of individual medusae on two different dates are given in Fish (1925), and in van der Maaden (1942) and Verwey (1942) for *C. capillata,* which give approximate instantaneous growth rates of 0.16, and 0.09–0.13, respectively. Average growth for *C. capillata* and *C. lamarcki,* combined (Dittrich, 1988), show that for them, $k = 0.02$–0.06, and for *C. lamarcki,* data in Russell (1931) give rates between 0.02 to 0.10 (average = 0.05). These instantaneous growth rates are generally within the range found in this study (0.02–0.13). Such values indicate that the mass of these medusae can double in 5 to 34 days (average = 12 days) and thus they are capable of exploiting fast-growing prey populations (*e.g.,* winter-spring zooplankton blooms) whenever they occur (Hernroth and Gröndahl, 1983; Larson, 1986a).

Initial maturation of *Cyanea* in the Niantic River, and the early movement of blastulae to their oral folds, is correlated with surface temperature. The effect of temperature may be indirect, affecting food source, or direct, affecting the metabolic activity of the medusae (*e.g.*, Heeger and Möller, 1987), but probably these and many other factors are involved with temperature serving as a surrogate for a complex of physical and biological interactions. However, this relationship is true only for larger medusae. Hamner and Jenssen (1974) indicate that maturation is size dependent in *Aurelia*, but they speculate that appropriate manipulation of the food supply might lead to precocious maturation of small individuals. If such a stimulus governs maturation in *Cyanea*, and is sufficient for large medusae, it seems reasonable that it is sufficient for all but the smallest individuals present at the same time. The physical or biological stimuli for maturation may be quantitatively or qualitatively different for large and for small medusae. If maturation were determined by a suite of conditions for which temperature is an index, all sizes of medusae should mature more or less simultaneously.

Maturation in *Cyanea* might also be decoupled from age of the medusa. The unimodal size frequency distributions exhibited by *Cyanea* in the Niantic River suggest that the small medusae which reproduce are more than a month older than larger specimens. These small individuals are unlikely to be large ones which have degrown, for the gonads rapidly and entirely regress in such medusae, so much so that they are difficult to biopsy (Hamner and Jenssen, 1974); even the smallest of these *Cyanea* (26 mm) are as easily sexed as are large individuals. It appears that all female medusae (which survive) in a given generation produce offspring. Plasticity in the size (and possible flexibility in the age) of reproducing individuals insures maximal reproductive output by the population regardless of ambient conditions, but how this is effected is not clear.

Planulae first appear on the oral folds of *Cyanea* at the time when the maximum average size of individuals is attained as is also true for *Aurelia* (Rasmussen, 1973; Dittrich, 1988), and after the maximum size is reached, the average size of *Aurelia* declines as it does in *Cyanea* (Rasmussen, 1973; Hamner and Jenssen, 1974; Kakinuma, 1975; Dittrich, 1988). Hamner and Jenssen (1974) and Dittrich (1988) indicate that decline in average size is associated with deterioration of medusae. Cargo (1974) mentions that *Chrysaora quinquecirrha* are found in a moribund state lacking tentacles or oral folds in nature for no apparent reason and thinks that the cause of deterioration merits study.

Deterioration may be induced by completion of embryogenesis to the blastula-planula stage as noted by de Lafontaine and Leggett (1988); the gonads of medusae in late stages of deterioration are devoid of germinal epithelium (pers. obs.), and there is no advantage for prolongation of life beyond this time. The temperature at which deterioration occurs ranges between 17° to 23°C, considerably less than that considered lethal to *Cyanea* (26.8°C) by Mayer (1914), and deterioration is neither size (nor age?) dependent as is also sexual maturation.

A functional relationship between deterioration of *Cyanea* and their reproduction has not been made in previous studies. That is, "spawning" (not defined, but probably meaning the appearance of blastulae or planulae on the oral folds) is followed by loss of tentacles and other body parts (Hoese *et al.*, 1964), or is followed by degeneration of feeding structures and death (Fancett, 1986); Berrill and Berrill (1981) simply say that medusae die after "spawning". Death might reasonably seem imminent when tentacles are lost, for the medusae can no longer feed. In *Cyanea*, the tentacles are lost early in the process of deterioration, while the planula-laden oral folds are still intact. Release of planulae and deterioration seem not to be sequential and separate events for *Cyanea*. Indeed, it appears that deterioration of *Cyanea*, and the loss of oral folds in particular, can be interpreted as a mechanism for deposition of most of its planulae (*e.g.*, Fig. 8) and in consequence is a normal extension of the reproductive process analogous to the post-reproductive parental role served by the death, detachment, and wind transport of tumbleweed (*Salsola pestifer*) in the disperal of seeds (Allee *et al.*, 1949).

Cyanea may provide transport for planulae to the benthos on its oral folds, though other medusae do so differently. For example, *Chrysaora* liberates gametes, with fertilization and development of planulae occurring in the plankton (Cargo, 1979); Cargo and Schultz (1967) found more-or-less continuous settling on suspended oyster shells from before August to the second week of September, as would be expected for planulae free in the water column. Also, *Aurelia* exhibits a pattern of planula settlement on plates suspended in the field similar to that for *Chrysaora* (Gröndahl, 1988) suggesting that the planulae are liberated as a free-swimming stage at intervals over an extended period (*e.g.*, Russell, 1970). These observations are consistent with finding mass occurrences of *Aurelia* planulae, and sporadic presence of *Chrysaora* planulae, in surface plankton (Widersten, 1968). In contrast, Widersten (1968) does not clearly indicate that planulae of *C. capillata* or *C. palmstruchi* (= *lamarcki*) are also found in plankton. If they are not, this might explain the observations of Gröndahl and Hernroth (1987) who report that no *Cyanea* settled on suspended plates from June to November, though their females with planulae were abundant; only once, late in the season (mid-November), did their planulae settle on such plates, and the medusae disappeared shortly thereafter (Gröndahl, 1988). This is expected if the planulae of *Cyanea* are not dispersed in the plankton as appears to be the case for other semaestomes, but rather are released, *en masse*, attached to oral folds lost from deteriorating medusa.

A problem with this interpretation is that healthy, intact medusae in a collecting bucket or in an aquarium "shed" planulae (and blastulae). To obtain polyps of *Cyanea* for observation in the field, Gröndahl and Hernroth (1987) and Gröndahl (1988) established aquaria containing female *Cyanea* along with settling plates which became colonized and could then be transported to field sites. In the confinement of an aquarium [or in a collecting bucket (pers. obs.)], the oral folds contact the surfaces of the container and the objects placed therein, and planulae are released under these conditions. However, that such contact is the stimulus freeing the planulae from the oral folds is difficult to test directly; while the null hypothesis potentially can be rejected, the alternative outcome is more likely (see above) but is ambiguous because to capture and transport medusae without their oral folds being disturbed or making surface contact is impossible. This interpretation that the oral folds of deteriorating medusae are a substrate for transport of planulae to the benthos relies on the field observations, above, and on the apparent absence of planulae in the water column. [It is unlikely that the appearance of planulocysts on shells are from planulae freely deposited some days, or weeks, earlier because the planula is short-lived (Lohman, 1908; Kaestner, 1969; Schneider and Weisse, 1985), and attach within a few hours (Brewer, 1984).]

The oral folds of *Cyanea* are sufficiently large, [33 ± 4% of total wet weight (unpubl.)] to accommodate the planulae it produces. It is a much-folded structure (Agassiz, 1862) with both an extensive surface area and a long margin. Measurements of the length of this margin on a small medusa (38 mm diameter) indicate that for each 10 mm of bell diameter the oral folds are 207 mm in length. Thus, if the same allometry holds, a 100-mm medusae has oral folds with a margin of about 2 m in length. This is more than sufficient to accommodate, in a band about 2 mm to 5 mm wide, the approximately 500,000 blastulae developing into planulae (200 mm × 100 mm), which a medusa of this size is conservatively estimated to produce (unpubl.). The accumulation of planulae along the margin of the oral folds is readily visible in reproducing specimens, and is of about these dimensions. The accumulated planulae also can be retained by the oral folds; von Lendenfeld (1884) reported that planulae develop almost to the polyp stage on the oral folds of *C. annaskala* (= *capillata*). The oral folds of other Scyphozoa may be proportionately as voluminous as those of *Cyanea*. For example, they are 18–38% of wet weight in *Chrysaora fuscescens* (Shenker, 1984), but their use for macrophagy in *Chrysaora* (Larson, 1986c) may be incompatible with the brooding of planulae, hence they release their gametes into the plankton. *Aurelia* does brood planulae (*e.g.*, Hargitt and Hargitt, 1910; Russell, 1970; Cargo, 1974), but their retention on the oral folds may be precluded by their small relative size [13 ± 4% of wet weight (unpubl.)].

The selective advantage accruing by deposition of planulae while still on the oral folds is not obvious. It would accelerate their descent to the benthos. The sinking rate of cnidarian planulae does not exceed 0.38 cm s^{-1}, and the swimming capacity of *Aurelia* planulae is even less, 0.042 cm s^{-1} (in Chia *et al.*, 1984). The sinking rate of developing embryos of *Chrysaora* is 0.01 cm s^{-1} (unpubl.). It is unlikely that the planulae of *Cyanea* would sink faster than these. In contrast, the sinking rate of oral folds is 1.4 ± 0.3 cm s^{-1} (unpubl.), one or two orders of magnitude more rapid than for isolated planulae. This, and the areal extent of the oral folds, undoubtedly alters the trajectory of descent and the extent of horizontal movement (in currents) in a way that perhaps is advantageous. In addition, transport to the benthos on the oral folds may facilitate the settlement of planulae by bringing them into more intimate contact with suitable substrate making it easier to penetrate the barrier of the boundary layer (*e.g.*, Vogel, 1981). Also, such transport could increase the likelihood of the successful development of earlier embryonic stages still in the confines of the oral folds which otherwise might not form normal planulae (Kakinuma, 1975).

The unexpected constancy of pattern and of numerical stability of *Cyanea* medusae in the Niantic River estuary [only 5.3×10^6 m^3 at MLW (Marshall, 1960)] suggests that the open water, neritic and oceanic, populations of *Cyanea* might be similar. In these even more stable environments, the wide swings in observed abundance may reflect more the vast volume in which they occur, and the currents to which they are exposed (Verwey, 1942; Shenker, 1984; Gröndahl, 1988), rather than representing inexplicable fluctuations in population size. Such regularity results from their rapid growth and efficient exploitation of a changing food supply, reproduction that is tied neither to size nor age, and on the consistent success in the transition to the benthic phase of its life history which could be effected by deposition of planulae while they remain on the oral folds of their nonfeeding, moribund parent.

Note: I have referred throughout this paper to *Cyanea* in the Niantic River estuary without giving a specific designation. There is a possibility that it is not *C. capillata* (*s.s.*) to which it unambiguosly keys (Larson, 1976; Shih, 1977). Its differences from typical *C. capillata* are comparable to those which distinguish *C. lamarcki* from *C. capillata* in British (Russell, 1970) and European (Gröndahl, 1988) waters. However, the general conclusions of this paper should apply at the generic level.

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Correlation of Histocompatibility Reactions with Fusion Between Conspecifics in the Solitary Urochordate *Styela plicata*

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Abstract. Previous transplantation analysis has identified a sensitive histocompatibility system in the solitary urochordate *Styela plicata* that obeys genetic transplantation "rules" identical to those of vertebrates. The current study demonstrates that histocompatibility acts to prevent fusion between conspecifics in sedentary aggregations of this species. Pairs of naturally fused individuals were present at low frequencies (0.06%) in natural populations. Comparative transplantation analysis, in which grafts were transferred from fused donors to panels of single (non-paired) recipients, confirmed that such fusion occurs only between individuals of identical histocompatibility tissue type. Ninety-four percent of recipients yielded identical responses to allografts from both members of fused pairs, suggesting that fused individuals expressed concordant histocompatibility tissue types. In contrast, only 69% of hosts receiving allografts from pairs of randomly selected unfused individuals yielded identical responses to both donor tissues. Allozyme analysis confirmed the specificity of this correlation between fusion and shared histocompatibility type. Fused individuals retained distinct genetic identities for electrophoretically resolved loci independent of tissue type.

In light of these results, it is argued that the role of histocompatibility systems in preventing fusion may have been a strong selective force in the evolution of allogeneic recognition.

Introduction

Invertebrate histocompatibility responses have frequently been implicated in the prevention of fusion between conspecifics (Bigger *et al.*, 1982; Buss *et al.*, 1984; Smith and Hildemann, 1984; Buss and Green, 1985; Kaye and Reiswig 1985; Van de Vyver *et al.*, 1985; Mukai and Shimoda, 1986). In colonial urochordates, allogeneic discrimination prevents fusion between incompatible colonies, but allows the integration of asexually derived zooids and other compatible individuals (Tanaka, 1973; Mukai and Watanabe, 1974; Katow and Watanabe, 1980; Taneda and Watanabe, 1982a, b). Despite this demonstrable regulatory function in recruitment among colonial species, the physiological role of histocompatibility in solitary urochordates has not been investigated.

However, recent evidence suggests that solitary species possess both the capacity for fusion *in situ* and sensitive histocompatibility mechanisms. Schmidt (1982) has shown that aggregative settlement in the solitary urochordate *Molgula complanata* results in the fusion of tunic matrices among 20% of metamorphosed adults. This finding complements tissue transplantation studies in *S. plicata*. In this species, approximately 75% of interpopulation tunic allografts undergo chronic rejection which subserves genetic transplantation "rules" identical to those of vertebrates (Raftos *et al.*, 1987a, 1988).

The aim of the current study is to correlate such observations by identifying a specific requirement for shared histocompatibility tissue type among individuals of *S. plicata* that have undergone fusion *in situ*. In doing so, the significance of conspecific fusion as a potential selective stimulus for allorecognition will be extended beyond that of specific adaptation to colonial life histories.

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Materials and Methods

Animals

Fifty-two pairs of putatively fused *S. plicata* specimens were collected from shark netting or pontoons at two sites on Sydney Harbor, Balmoral Beach, and Birkenhead Point, Australia. These sites were separated by 8 km of estuarial coastline. Both areas maintained large, stable populations of *S. plicata*. Putatively fused pairs were initially identified *in situ* as individuals living in close association such that large areas of their tunics were in direct contact. The frequency of these pairs, relative to single (unpaired *in situ*) animals at the Birkenhead Point site, was determined as a function of mean population density based on counts of all individuals in 10 randomly selected quadrats. An additional 384 single Balmoral Beach individuals were collected for use as controls or graft recipients.

During the carmine retention studies and prior to grafting, specimens were held in 30-l polyethylene aquaria under constant aeration and filtration. After grafting, recipients were cultured *in situ*. They were enclosed in plastic mesh cages secured to the 0.1 m tide level on shark netting at Balmoral Beach.

Morphological analysis of fusion

The identification of individuals exhibiting fusion was confirmed by removing 1 cm^3 blocks of tunic tissue incorporating the interface between putatively fused animals. For comparison, similar blocks were removed from the epidermal layer of 20 single (unpaired *in situ*) individuals. Tissue blocks were fixed for 24 h in formalin (10% v/v in filtered seawater) and then sectioned (10 μm) with a freezing microtome (Cryostat, Massachusetts). Sections were stained with haemotoxylin and eosin (Humason, 1972) prior to bright field microscopical inspection. Based on this examination, "fused" pairs were defined as those lacking part or all of an eosinophilic layer at their point of contact (Fig. 1). Closely associated individuals which retained a continuous eosinophilic boundary at the interface were defined as "nonfused" pairs (Fig. 1).

Further resolution of tunic fusion was provided by scanning electron microscopy (SEM). The blocks of tunic tissue remaining after sectioning for bright field analysis were washed by immersion in filtered seawater for 24 h before being critical point dried using liquid CO$_2$ as the transition medium. Dried tissue was mounted on aluminum stubs with the microtome cut surface facing upwards and then sputter coated with gold (4 min, 20 mA). Specimens were inspected with a Joel 840 scanning electron microscope at an accelerating voltage of 10 kV. (Joel Australasia Ltd., Sydney).

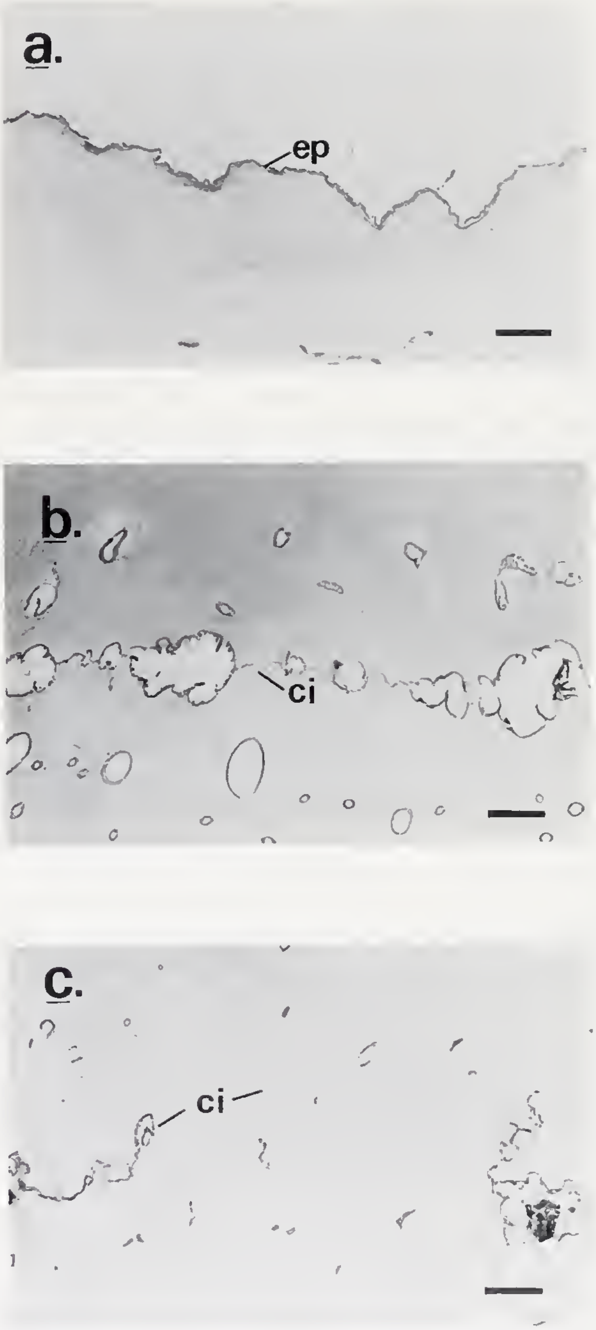

Figure 1. Bright field micrographs of the: (a) outer epidermis of a single (unpaired) individual. (b) contact interface between two "nonfused" animals. (c) contact interface between "fused" individuals. ci = contact interface, ep = epidermis. Scale bars = 1 mm.

Analysis of vascular connection

The degree of vascular connection between closely associated individuals was initially investigated by bright field microscopical analysis of frozen sections. The frequency of tunic vessels was determined within 5 mm of the contact interface between putatively fused animals and within 5 mm of the epidermis of single (unpaired *in situ*) animals.

Vascular interaction was further delineated by the transmission of particulate carmine between fused individuals. One animal from each of four fused and four non-fused pairs (as defined above) was injected with 400 μl carmine (10% w/v in filtered seawater). Carmine solutions were injected into blood sinuses of the mantle lying directly beneath the tunic. Injected animals were subsequently maintained in aquaria. To identify any passive uptake of excreted carmine, single control animals were placed with the injected pairs in each aquarium.

Three days after injection, 200 μl samples of hemolymph were removed from scalpel wounds made in the right posterior tunic and mantle of both animals from each pair and control individuals. The frequency of carmine particles in these samples was determined using a hemocytometer.

Comparative histocompatibility tissue typing

The relationship between fusion and histocompatibility was assessed by comparative tissue typing based on established tunic transplantation techniques (Raftos *et al.*, 1987a, b, 1988) and previously defined genetic "rules" for transplantation rejection in *S. plicata* (Raftos *et al.*, 1988; Raftos and Briscoe, 1989). Transplants, in the form of cylindrical cores, were removed from the tunic of donor individuals by obliquely inserting a length of 3 mm diameter stainless steel syringe tubing. Grafts were implanted into holes in the tunic of the host that had been prepared by similar insertion of syringe tubing and removal of detached cores. This procedure was used to provide two forms of graft. Autografts were transferred within a single individual so that they assumed a position 1 cm anterior to their original location on the dorsal stolonic margin. In contrast, allografts represented tissue orthoptically transferred between separate individuals.

The relative tissue types of fused individuals were determined by transplanting allografts from nine fused pairs to panels of randomly chosen single Balmoral Beach individuals. Each animal in the recipient panels received two simultaneous allografts, one from each member of a particular fused pair. The two allografts were separated from each other by at least 1 cm. The size of the recipient panel derived from each fused pair was dependent upon the number of grafts that could be extracted from the donors. Fused pairs usually provided sufficient grafts for twenty recipients.

This tissue typing procedure was repeated using single animals as donors to provide estimates of tissue type concordance in the general (unfused) population. In these trials, 20 single Balmoral Beach individuals were randomly allocated into 10 donor pairs, providing allografts for panels of approximately 10 recipients per pair.

Estimates of the frequency for graft loss arising from technical failure of the transplantation procedure were obtained by implanting dual autografts into a further 60 single Balmoral Beach animals.

The viability of grafts was determined 65 days after grafting. Assessment at this time provided an accurate distinction between compatible and incompatible grafts (Raftos *et al.*, 1987a). Mortality among grafted animals was such that graft viability could be assessed in 81, 68, and 43 individuals that had received tissue from fused pairs, paired single animals, or autogeneic combinations, respectively. Tissue incompatibility was defined as the complete rejection of the graft from the graft bed, whereas compatibility was characterized as the retention of some or all of the graft. Recipients were deemed to have yielded a concordant response to transplantation if both grafts from separate donors were retained (++) or rejected (−−). Non-concordant responses occurred when a graft from one donor was retained, while that from the other donor was rejected (+−).

Electrophoretic analysis of genetic variation

The similarity of fused individuals for factors other than histocompatibility was determined electrophoretically. Gonads and pharyngeal baskets were removed from both animals in nine fused pairs, and from 18 single Balmoral Beach animals that had been randomly allocated into pairs. Tissue samples were homogenized in 0.1 *M* phosphate buffer and subjected to starch gel electrophoresis (Harris and Hopkinson, 1976). Gels were stained for the enzymes: phosphoglucose isomerase, E.C. 5.3.1.9. (PGI); aspartate aminotransferase, E.C. 2.6.1.1. (AAT); and phosphoglycerate kinase, E.C. 2.7.2.7. (PGK-2), by the methods of Richardson *et al.* (1986).

Results

Morphological analysis of fusion

Based on histological analysis of the contact interface, the 52 putatively fused pairs identified *in situ* fell into two distinct groups. Eighty-three percent of putatively fused animals failed to exhibit fusion of their tunic matrices, and thus were considered to be closely associated but "non-fused" pairs. Bright field inspection of frozen sections revealed an eosinophilic layer at the contact interface in these non-fused pairs (Fig. 1b). SEM resolved this layer as a dense tunic boundary (Fig. 2b) similar to the proteinaceous (Goodbody, 1974) epidermal layer of sin-

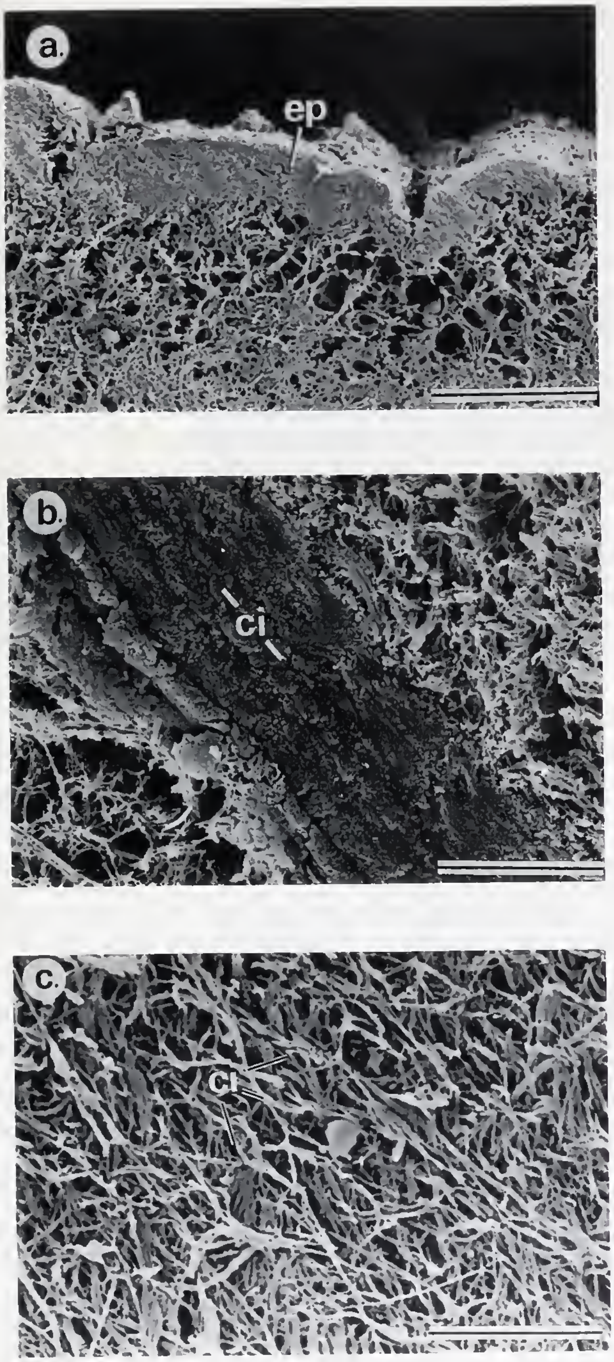

Figure 2. SEM micrographs of: (a) the epidermis of a single animal. (b) Dense tunicin boundaries at the contact interface between individuals in a "non-fused" pair. (c) Interdigitation of tunicin fibres at the contact interface between "fused" animals. ci = contact interface; ep = epidermis. Scale bars = 10 μm.

gle animals (Figs. 1a, 2a). The boundary prevented the interdigitation of extracellular tunic fibers between individuals, allowing animals in non-fused pairs to be pried apart with little effort.

The remaining 17% of putatively fused individuals identified *in situ* exhibited an obvious fusion of their tunic matrices. In these "fused" pairs, eosinophilic boundaries were either intermittent or absent from the point of contact (Fig. 1c). This absence of dense boundaries between fused animals was reflected in SEM by the interdigitation of tunicin fibers between individuals (Fig. 2c) yielding a confluent tunic matrix.

Fusion was most often associated with a unique external morphology (Fig. 3). Contact was developed, between individuals of approximately equal size, along the ventral stolonic margin, so that the atrial siphons were not adjacent. Such fused animals could not be pried apart without causing extensive damage to the tunic. Based on the mean population density at the Birkenhead Point collection site, the relative frequency of fused pairs conforming to these characteristics was 6.4×10^{-4}.

Vascular connection of fused animals

The distinction between fused and non-fused pairs was further delineated by the penetration of vascular elements into the contact zone between individuals. Figure 4 reveals that non-fused pairs exhibited a similar pattern of vascular penetration to that of the epidermis in single (unpaired *in situ*) animals. The maximum frequency of tunic vessels occurred 3–5 mm from the contact zone in non-fused pairs and fell to 0 at the interface, indicating a lack of vascular connection between animals. In contrast, pairs exhibiting fusion of tunic matrices showed peak tunic vessel frequencies closer to the point of contact (1–2 mm) and retained substantial vascular activity at the interface, confirming vascular connection between fused animals.

The fate of injected carmine particles indicated that this vascular connection of fused pairs allowed substantial interchange of hemolymph components. In fused pairs, 21 ± 2% of the carmine particles that had entered the hemolymph of injected animals were transferred to the second, uninjected individual. In contrast, uninjected animals in non-fused pairs accumulated only 0.8 ± 0.4% of the carmine found in the hemolymph of their partners. This did not differ significantly ($t_6 = 0.91$; $P > 0.05$) from the frequency of carmine in the hemolymph of uninjected single control animals that were cultured in the same aquaria as injected individuals. In these control individuals, minimal uptake of carmine occurred either through phagocytic ingestion of excreted carmine or via penetration of the atrium during bleeding.

Comparative histocompatibility tissue typing

The relationship between histocompatibility tissue type and fusion is shown in Table 1. If, as postulated,

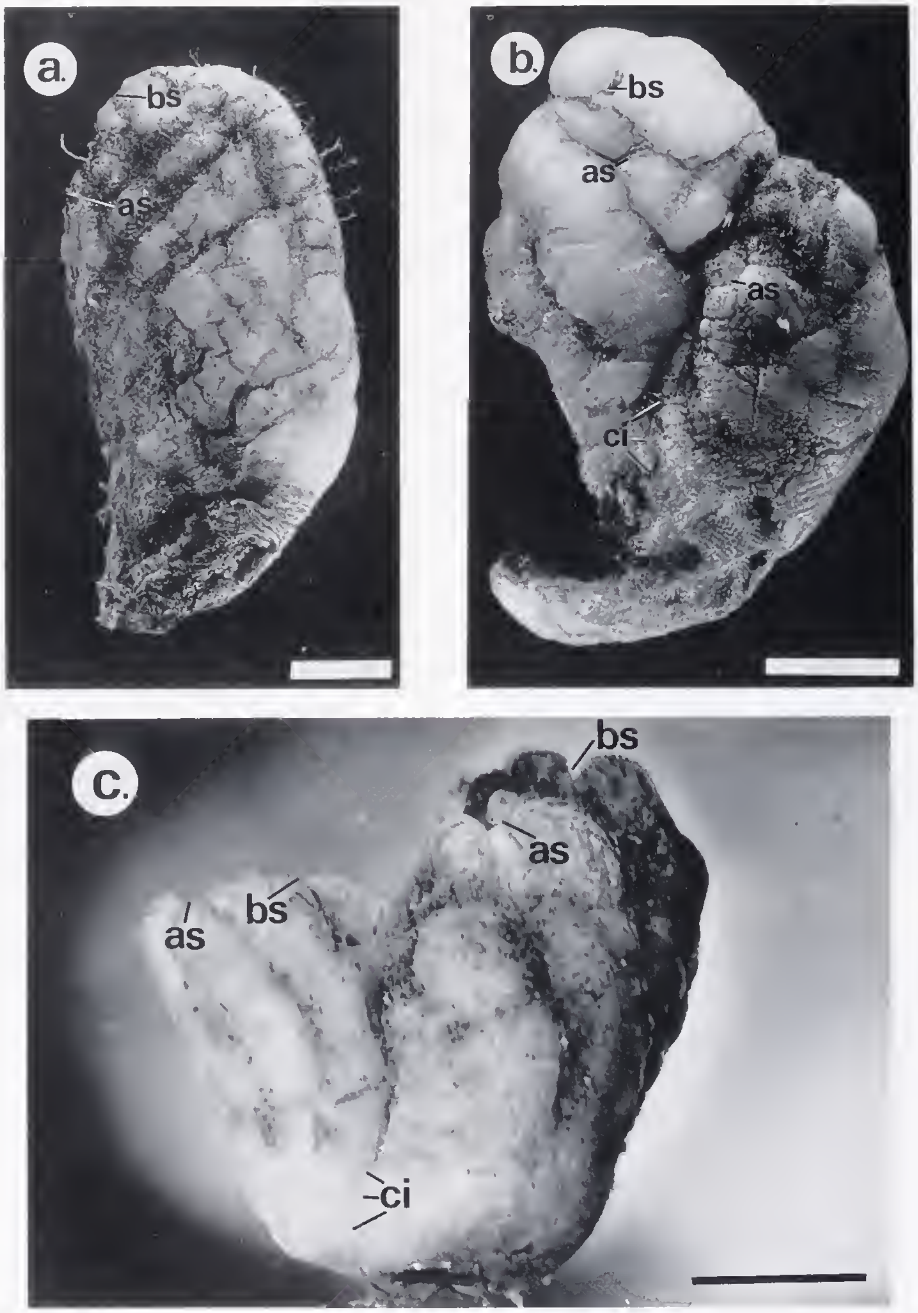

Figure 3. Typical external morphologies of: (a) single (unpaired) individuals. (b) "Non-fused" paired animals. (c) "Fused" individuals. as = atrial siphon, bs = buccal siphon, ci = contact interface between individuals. Scale bars = 1 cm.

fused individuals shared identical tissue types, it would be expected that recipients should yield identical, or concordant, responses to grafts from both members of a particular fused pair. Such expectations are based on previous genetic analysis which has shown that *S. plicata* obeys transplantation "rules" identical to those of vertebrates (Raftos *et al.*, 1988). Recognition of a single disparate histocompatibility haplotype between individuals will initiate graft rejection. Hence, to fuse, individuals should be of identical genotype with respect to histocompatibility and so should invoke concordant responses among panels of naive recipients in tissue typing trials. The data presented in Table I suggests that this is, indeed, the case. Ninety-four percent of individuals receiv-

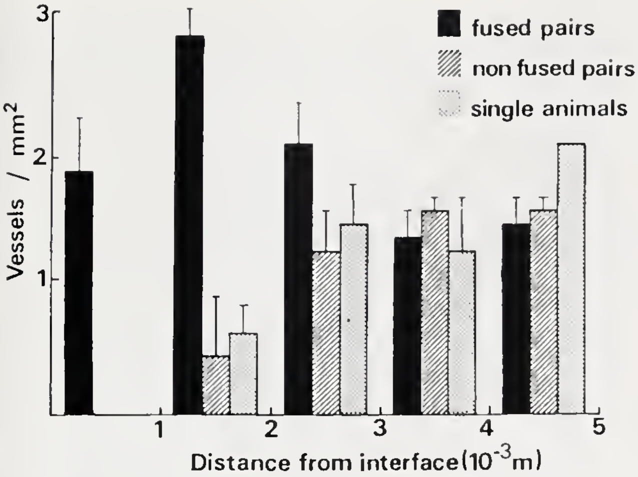

Figure 4. Mean frequencies of blood vessels at various distances from the contact interface between "fused" individuals or "non-fused" animals, or from the epidermis of single (unpaired) animals. Bars represent standard error ($n \geq 7$).

ing allografts from fused pairs exhibited concordant transplantation responses. This was characterized by simultaneous retention (++) or rejection (−−) of grafts from both members of a donor pair. There was no significant difference ($\chi^2 = 1.06$, $P > 0.05$) between the frequency of non-concordant (+−) results among animals returning +− or ++ responses toward fused pairs (27.8%) and that observed for autografts (16.2%). Taking this favorable comparison with non-specific autograft loss into account, concordance toward fused pairs approached 100%, indicating an identity of donor tissue types.

In contrast, 30.8% of grafts from paired single animals returned non-concordant (+−) responses in recipient panels. This was significantly greater than the non-concordance frequency from fused pairs ($\chi^2 = 15.67$; $P < 0.05$). However, it corresponded ($\chi^2 = 2.98$; $P > 0.05$) with expected levels for a random binomial association of rejection and compatibility calculated from the total frequency for rejection (q; 0.71), such that randomly occurring non-concordance represented $\{2q(1-q)\} \times 100 = 41.2\%$.

Electrophoretic analysis of genetic variation

The similarity of electrophoretic genotypes between fused and single individuals is summarized in Table II. Electrophoretic analysis of three independent enzyme loci failed to reveal an overall genetic similarity between fused animals for factors other than histocompatibility that may have arisen through physiological chimerism or sibship. In 23% of fused pairs, individuals shared no allele at a single locus, while 41% shared one allele. Only 36% of fused individuals were genotypically identical at a given locus, and none shared identity at all three loci simultaneously. This pattern for fused pairs did not differ significantly ($\chi^2 = 1.06$; $P > 0.05$) from the level of genotypic similarity observed between pairs of randomly chosen single animals.

Table I

Frequency of concordant (++ or −−) and non-concordant (+−) transplantation reactions in individuals of Styela plicata *that had received either two allografts, one from each member of a fused pair, or two allografts from separate randomly paired single animals, or two autografts*

	Frequency (%)				
	Graft status[α]				
Donor animals	++	+−	−−	Total[β] rejection	Total concordance
Fused pairs (n[δ] = 81)	16.0	6.1	77.7	80.8	93.8
Paired single animals (n = 68)	13.2	30.8	55.8	71.3	69.1
Autografts (n = 43)	83.7	16.2	0.0	8.1	83.7

[α] ++, Denotes both grafts from separate donors accepted; +−, graft from only one donor intact; −−, grafts from both donors rejected.

[β] Total number of grafts rejected divided by the total number implanted.

[δ] Number of graft recipients scored 65 days after grafting.

Discussion

The data presented here suggest that fusion will occur between conspecifics of *S. plicata* only when the individuals share common histocompatibility tissue types. Fu-

Table II

Similarity of electrophoretic genotypes for individuals in fused pairs and randomly selected pairs of single (unfused) animals

	Frequency (%)[α]		
Type of pair	No common allele	One common allele	Identical genotype
Fused pairs (n[β] = 9)	23	41	36
Single animals (n = 9)	23	27	50

[α] Data represent totals of independently determined frequencies for three different enzyme loci (PGI, AAT and PGK).

[β] Number of pairs tested.

sion in the natural environment was found to be a rare event involving a physiological confluence of fused animals. The eosinophilic epidermal layer observed in single animals, and between non-fused pairs, was absent from the contact interface between individuals in fused pairs. This allowed a vascular connection between fused individuals, exemplified by the penetration of tunic vessels across the contact interface. The exchange of particulate carmine between fused animals confirmed that this vascular penetration represented a functional connection.

It is postulated that such fusion is permitted by the sharing of histocompatibility tissue types between individuals. According to this hypothesis, the frequency of concordant response by recipient animals to grafts implanted from both members of a fused pair should approach 100%, reflecting identity of donor tissue types. Cases of non-concordant response should not occur if fused donors were of identical tissue type. Comparative histocompatibility tissue typing in this study has fulfilled these constraints. Concordant responses were recorded from 94% of animals receiving grafts from fused pairs. Taking into account the level of technical graft loss reflected by control autografts, this result approaches the predicted 100% frequency of concordance. In contrast, 31% of the individuals receiving grafts from arbitrarily paired single animals returned non-concordant responses consistent with a random association of rejection and compatibility. Clearly, grafts from single (unpaired *in situ*) animals exhibited independent histocompatibility tissue types, yielding a high frequency of non-concordant responses, while those from fused pairs shared histocompatibility types, and thus were subject to identical reactions.

The demonstrated correlation between tissue type and fusion may be explained by three alternative hypotheses. First, histocompatibility mechanisms may act to specifically prevent fusion. In contrast, fused individuals may share tissue types as a result of genetic chimerism arising from fusion. Finally, fusion may preferentially occur between animals of general genetic similarity (*i.e.*, sibs) and thus would reflect greater levels of tissue compatibility than the normal population. The electrophoretic analysis undertaken in this study supports the former option, that histocompatibility mechanisms specifically prevent fusion. Fused animals retained distinct genetic identities for characteristics other than histocompatibility tissue type. Their level of genetic similarity at three enzyme loci did not differ significantly from that of paired single animals. Thus, the observed correlation between histocompatibility and fusion could not have arisen from a broad genetic similarity derived from the development of genetic chimerism through physiological connection and is also unlikely to reflect sibship among fused animals. However, these results do not preclude a strong linkage disequilibrium between gene regions controlling histocompatibility and some other factor responsible for fusion.

The requirement for similarity of tissue type in fusion confirms that at least one function of the histocompatibility system in this sessile aggregative species is the prevention of fusion between conspecifics. Clearly though, histocompatibility is not the only factor preventing fusion. Previous genetic analysis has indicated that 9.5% of individuals in natural populations of *S. plicata* share histocompatibility tissue types, and so have the potential for fusion (Raftos and Briscoe, 1989). A far lower frequency of fused pairs was identified in the current study (0.06%). When taken with the consistent morphology of fused pairs, this suggests that other factors, such as the proximity, timing, and orientation of larval settlement, may play a substantial role in fusion. However, the paucity of fused pairs may also attest to the deleterious nature of fusion, reflecting a selective stimulus for the development of histocompatibility. It has been suggested that such selection pressure in invertebrates arises from germ or somatic cell parasitism, by which the individual genetic identity of fused animals may be eliminated (Buss and Green, 1985; Buss, 1982; Rinkevich and Weissman, 1987). However, electrophoretic analysis in this study has failed to identify a loss of genetic integrity in either gonadal or somatic tissue, even though substantial vascular connection was observed between fused animals. An alternative explanation is that fusion greatly increases the likelihood of infectious disease through the transfer of pathogens between individuals.

Regardless of its precise selective basis, the experimental correlation between fusion and histocompatibility in *S. plicata* remains clear. It suggests that the physiological functions of histocompatibility in invertebrates extend beyond the regulation of life histories in colonial organisms, and so may represent a generalized selective factor in the development of specific immunological recognition.

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Microscopical, Biochemical, and Immunological Studies of the Immune Defense System of the Horseshoe Crab, *Limulus polyphemus*

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Abstract. The immune defense system of *Limulus polyphemus* was explored. Two distinct cells, granulocytes and plasmatocytes, were revealed in the hemolymph by light microscopy of live cells, by light microscopy serial sectioning, and by transmission electron microscopy combined with immuno-gold labelling. The granulocytes (amebocytes) comprised about 99% of the hemocytes. They had a heterochromatic nucleus, distended but poorly developed RER, few free ribosomes, few mitochondria, but many large secretory granules. While the majority of these were uniformly stained, structured granules were also present. Monoclonal antibodies revealed that coagulogen is present in both types of large secretory granules. It is suggested that a structured granule is an immature stage leading to a uniformly stained granule. The plasmatocytes had an euchromatic nucleus, extended RER consisting of tubular or flattened cisternae, many free ribosomes, many mitochondria, but few, if any, large secretory granules. Coagulogen was not detected in plasmatocytes. Following immunizations of five rabbits for more than one year with cell-free hemolymph, 14 polypeptides were immunogenic in rabbits as visualized by crossed immunoelectrophoresis. However, O'Farrell gel electrophoresis combined with silver staining identified more than 70 major polypeptides in the cell-free hemolymph. At least 60 of these had pI-values between 7.5 and 5.5, and Mr-values from larger than 200×10^3 to smaller than 21×10^3.

Introduction

The four recognized extant species of horseshoe crabs are "living fossils," whose morphology has remained unchanged for about 200 million years (Sekiguchi and Sugita, 1980). If this stability is reflected in their physiology, studies of their immune defense system may reveal some of the molecular mechanisms responsible for their success in an environment of gram negative bacteria (Bang, 1956). Furthermore, such studies may eventually facilitate our understanding of how the immune defense system evolved in higher and more recent phyla than horseshoe crabs.

Introduction of gram negative bacteria into the hemolymph of *Limulus polyphemus* results in clotting (Bang, 1956). This coagulation is a result of extensive exocytosis from the hemocytes (Levin and Bang, 1964). In intact

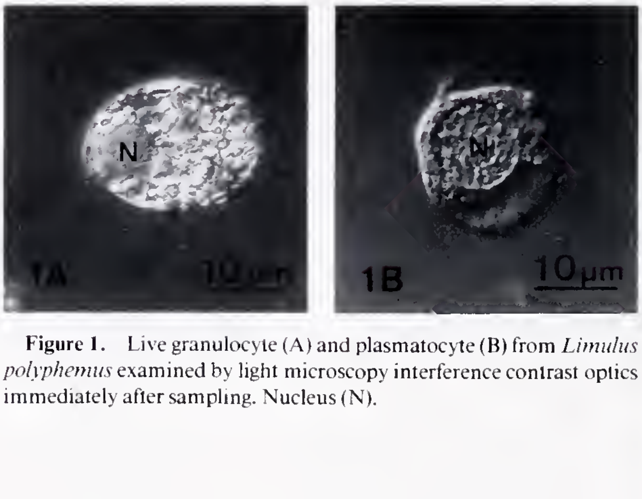

Figure 1. Live granulocyte (A) and plasmatocyte (B) from *Limulus polyphemus* examined by light microscopy interference contrast optics immediately after sampling. Nucleus (N).

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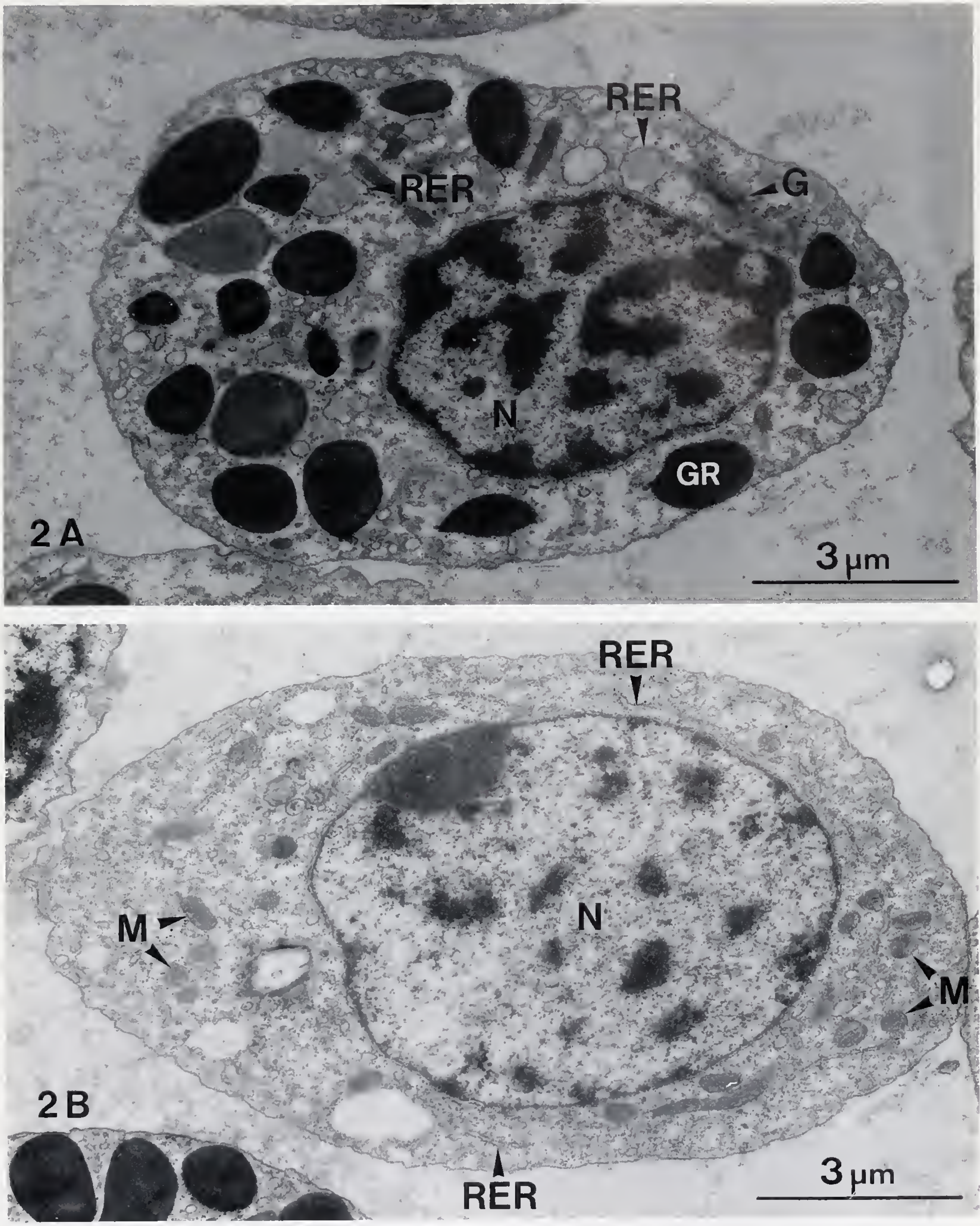
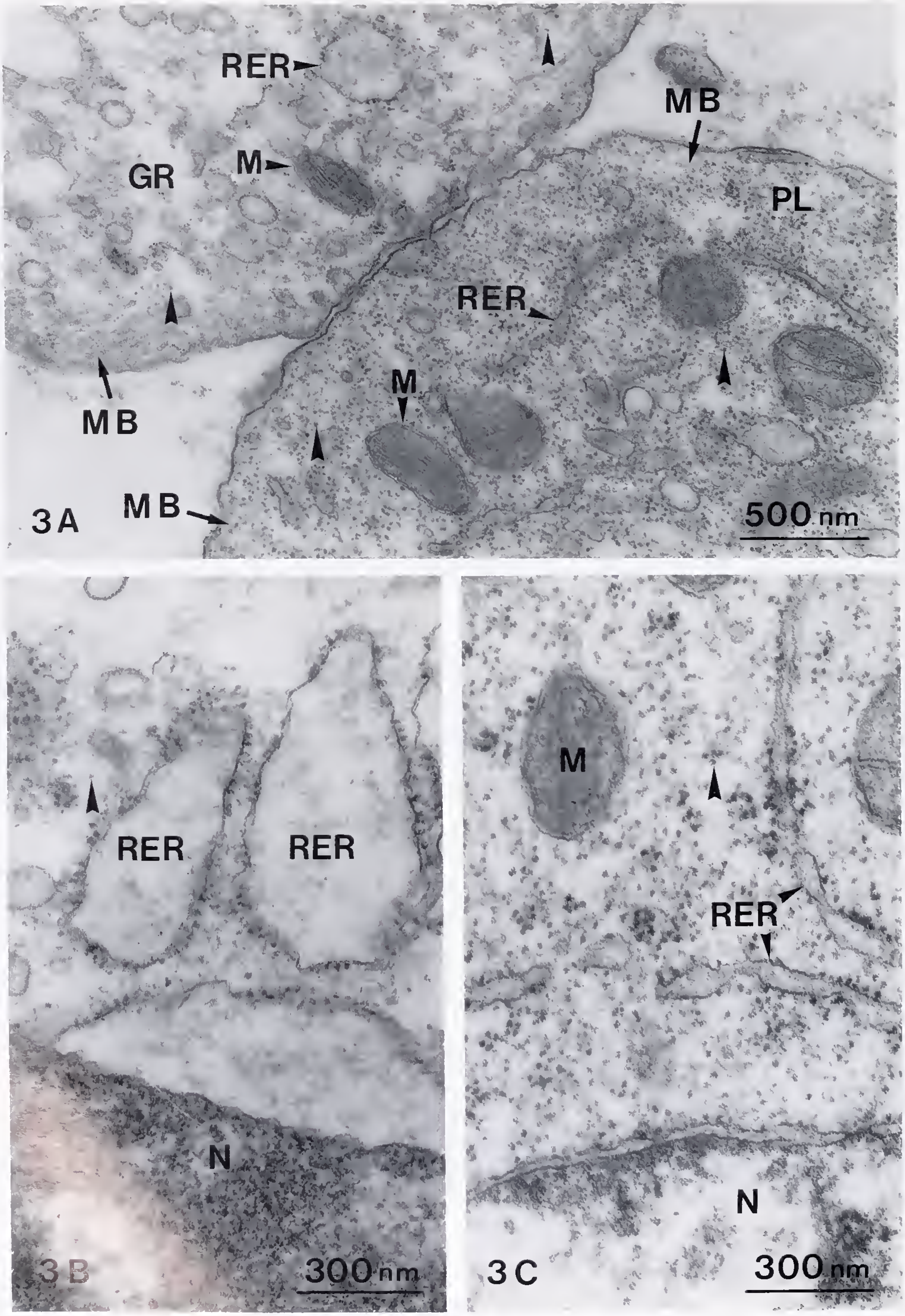

RER
RER
G
N
GR
2 A
3 μm
RER
M
N
M
2 B
3 μm
RER

Figure 2. Granulocyte (A) and plasmatocyte (B) from *Limulus polyphemus* examined by TEM. Golgi complex (G), large uniform dense secretory granule (GR); mitochondria (M); nucleus (N); rough endoplasmic reticulum (RER).


RER
MB
GR
M
PL
RER
M
MB
MB
3A
500 nm
RER
RER
N
3B
300 nm
M
RER
N
3C
300 nm

animals a tiny fraction of the hemocytes may be secreting spontaneously, while stimulation with gram negative bacteria or their lipopolysaccharides (LPS) induces a burst of exocytosis in the secretory cells (Armstrong, 1979; Ornberg, 1985; Iwanaga *et al.*, 1986). Lysates of hemocytes are now extensively used to detect minute quantities of LPS (Watson *et al.*, 1987).

The horseshoe crabs *L. polyphemus* and *Tachypleus tridentatus* have been reported to contain a single and two granular hemocytes, respectively (Dumont *et al.*, 1966; Shishikura *et al.*, 1977; Shishikura and Sekiguchi, 1979; Copeland and Levin, 1985). Cyanoblasts and cyanocytes are also evident in the general circulation of *L. polyphemus* (Fahrenbach, 1970; Sherman, 1981). They are characterized by their large size (diameter up to 100 μm) and their content of cytoplasmic hemocyanin crystals.

Recently we investigated the fine structure of unstimulated hemocytes from *T. tridentatus* using transmission electron microscopy (TEM) and light microscopy (LM) serial sectioning (P. P. Jakobsen, and P. Suhr-Jessen, unpub.). We found two populations of cells: granulocytes with a heterochromatic nucleus, many large secretory granules, and a poorly developed RER; and plasmatocytes with an euchromatic nucleus, few large secretory granules, and a well-developed RER. Cyanoblasts or cyanocytes were not observed. These observations prompted us to reinvestigate *L. polyphemus*, and here we report on the presence of granulocytes and plasmatocytes in this species too.

Few polypeptides have so far been discovered in the cell-free hemolymph. They include hemocyanin, an alfa 2-macroglobulin-like protease inhibitor, hemagglutinins, and lectins (Nachum *et al.*, 1979; Pistole, 1979; Cohen *et al.*, 1984; Pistole and Graf, 1984; Kempter *et al.*, 1985; Armstrong, 1985; Aketagawa, 1986; Liu *et al.*, 1987). Two-dimensional gel-electrophoresis combined with silver staining has previously identified an unforeseen high number of plasma and cerebrospinal fluid polypeptides in other organisms (Anderson and Anderson, 1977; Merrill *et al.*, 1981; Suhr-Jessen and Rasmussen, 1988). In *L. polyphemus*, these methods reveal more than 70 polypeptides in the cell-free hemolymph, among which only 14 are immunogenic in rabbit.

Materials and Methods

Animals

L. polyphemus (prosoma width: 4–22 cm) was purchased from the Marine Biological Laboratory, Woods Hole, Massachusetts, and kept at 15°C in The Salt Water Aquarium of Funen in seawater or in artificial seawater containing 3.0% NaCl. Hemolymph was drawn by cardiac puncture at the ethanol cleaned prosoma-opistosoma junction using a 19 gauge needle alone or combined with a 5 ml syringe. Cell-free hemolymph was prepared as described (Tvede and Baek, 1983). Samplings from all animals gave similar results.

Light microscopy of live cells

One part hemolymph was instantly diluted into one volume endotoxin free 3% NaCl containing 2 m*M* propranolol (Murer *et al.*, 1975). Samples were placed between alcohol cleaned object and cover glasses, examined using interference contrast optics and immersion oil (Leitz; 400×), and photographed.

Light- and transmission electron microscopy of dead cells

One part hemolymph was instantly mixed with 4 parts 5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4 for 30 min at room temperature followed by 90 min at 4°C, post fixed with 2% OsO_4, contrasted with uranyl acetate, and embedded in araldite (Willumsen *et al.*, 1987). Samples were sectioned on a Reichert OM U2 ultramicrotome. For light microscopy (LM), sections (about 500 nm) were stained with toluidine blue and examined in a Leitz microscope at 400×. For transmission electron microscopy (TEM), sections (about 50 nm) were mounted on pioloform F-50 coated Ni-grids, contrasted with 0.4% lead citrate in water for 5 min, and examined in a Jeol JEM-100CX electron microscope at 80 kV.

Immuno gold labelling

One part hemolymph was instantly mixed with 4 parts 2.5% glutaraldehyde plus 3.75% paraformaldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4, and left for 60

Figure 3. A comparison of granulocytes and plasmatocytes from *Limulus polyphemus*. (A) Granulocyte (GR) and plasmatocyte (PL). Free ribosome (arrow head); marginal band of microtubules (MB); mitochondria (M); rough endoplasmatic reticulum (RER). (B) Part of granulocyte with the heterochromatic nucleus, distended RER and few free ribosomes. Nucleus (N), otherwise symbols as in A. (C) Part of plasmatocyte with the euchromatic nucleus, many free ribosomes, and flattened or tubular RER. Symbols as in B.

Table I

A comparison of the abundance of mitochondria in plasmatocytes (PL) and granulocytes (GR) determined in three independent, complete serial sections

Serial section	Number of mitochondria in		Ratio PL/GR
	Plasmatocyte	Granulocyte	
# 1	203	61	3.33
# 2	364	120	3.03
# 3	318	114	2.79
Total	885	295	3

min at room temperature. It was then washed in cacodylate buffer (4 × 5 min), embedded in 2% agar, dehydrated in ethanol at gradually decreasing temperatures, and embedded in Lowicryl K4M according to manufacturer's guide. Formation of the ultra pyramid, sectioning, and incubation with antibodies were made the same day, since prolonged exposure to air seems to inactivate the antigens.

Incubations with antibodies were made by successively floating the grids on drops of (1) swine pre-immune serum (15 min), (2) monoclonal mouse anti-coagulogen antibody (120 min), (3) 0.05 *M* Tris buffer, pH 7.4 (3 × 5 min), (4) swine anti-mouse antibodies coupled to 10 nm gold particles (DAKOPATTS, 60 min), and (5) Tris buffer as above. While pre-immune serum was used directly, anti-coagulogen and gold-coupled antibodies were immediately diluted 50 fold with Tris buffer in the absence and presence of 2% fish gelatine (Sigma), respectively (Behnke *et al.*, 1986), before use. Samples were then contrasted in 1% uranyl acetate (10 min) followed by 0.4% lead citrate (5 min), and examined by TEM as described above.

Antibody production

To generate polyclonal antibodies against cell-free hemolymph, five rabbits were injected intradermally with 100 μl of cell-free hemolymph and an equal volume of Freunds incomplete adjuvant (Harboe and Ingild, 1973). The first 5 injections were spaced 14 days apart, and they were followed by monthly booster vaccinations for 6 months before the first bleeding. Thereafter, the immunization continued for more than 12 months. One week after each vaccination, 40 ml of blood was sampled from each rabbit. Serum from the five rabbits were pooled, and immunoglobulin G was purified and concentrated (Harboe and Ingild, 1973).

The generation of mouse monoclonal antibodies against coagulogen is described elsewhere (Zhang *et al.*, 1988).

Crossed immunoelectrophoresis

The immunoelectrophoresis was performed on 100 × 100 mm gels according to Weeke (1973). Five μl cell-free hemolymph was separated in the first dimension. The second dimension contained polyclonal rabbit antibodies against cell-free hemolymph (5 $\mu l/cm^2$ gel).

Polyacrylamide gel electrophoresis

Freshly drawn hemolymph was immediately centrifuged (5.000 g/4 min/20°C). The upper two thirds of the cell-free hemolymph was quickly diluted into five volumes of fresh lysis buffer (5 m*M* Tris pH 6.8, 2.5% SDS, 10% 2-mercaptoethanol, 1 m*M* PMSF), and boiled 4 min. Samples were then dialyzed at 4°C against double-distilled water, and freeze-dried. Lyophilized cell-free hemolymph was separated on two-dimensional 1 mm 10–16% polyacrylamide gradient gels and silver stained (O'Farrell, 1975; Merrill *et al.*, 1981; Suhr-Jessen *et al.*, 1986). This allowed the detection of less than 1 ng of each of the molecular weight markers myosin, beta-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (Mr-values of 200, 116, 92, 66, 45, 31, 21.5, and 14.4 × 10^3, respectively).

Results

General morphology of hemocytes

The hemocytes of *L. polyphemus* were spheroid and usually 15–20 μm at their longest axis (Figs. 1, 2, and 5). They contained a single non-lobated nucleus with one or a few nucleoli, RER, Golgi-complexes, mitochondria, and a marginal band of microtubules. Centrioles were present, but no signs of mitosis were seen.

Table II

A comparison of the main differences between plasmatocytes and granulocytes

	Plasmatocyte	Granulocyte
Nucleus	euchromatic	heterochromatic
RER	flattened and well developed	distended but poorly developed
Free ribosomes	many	few
Large secretory granules	few—if any	many
Coagulogen	no	yes
Mitochondria	many	few
Frequency	1%	99%

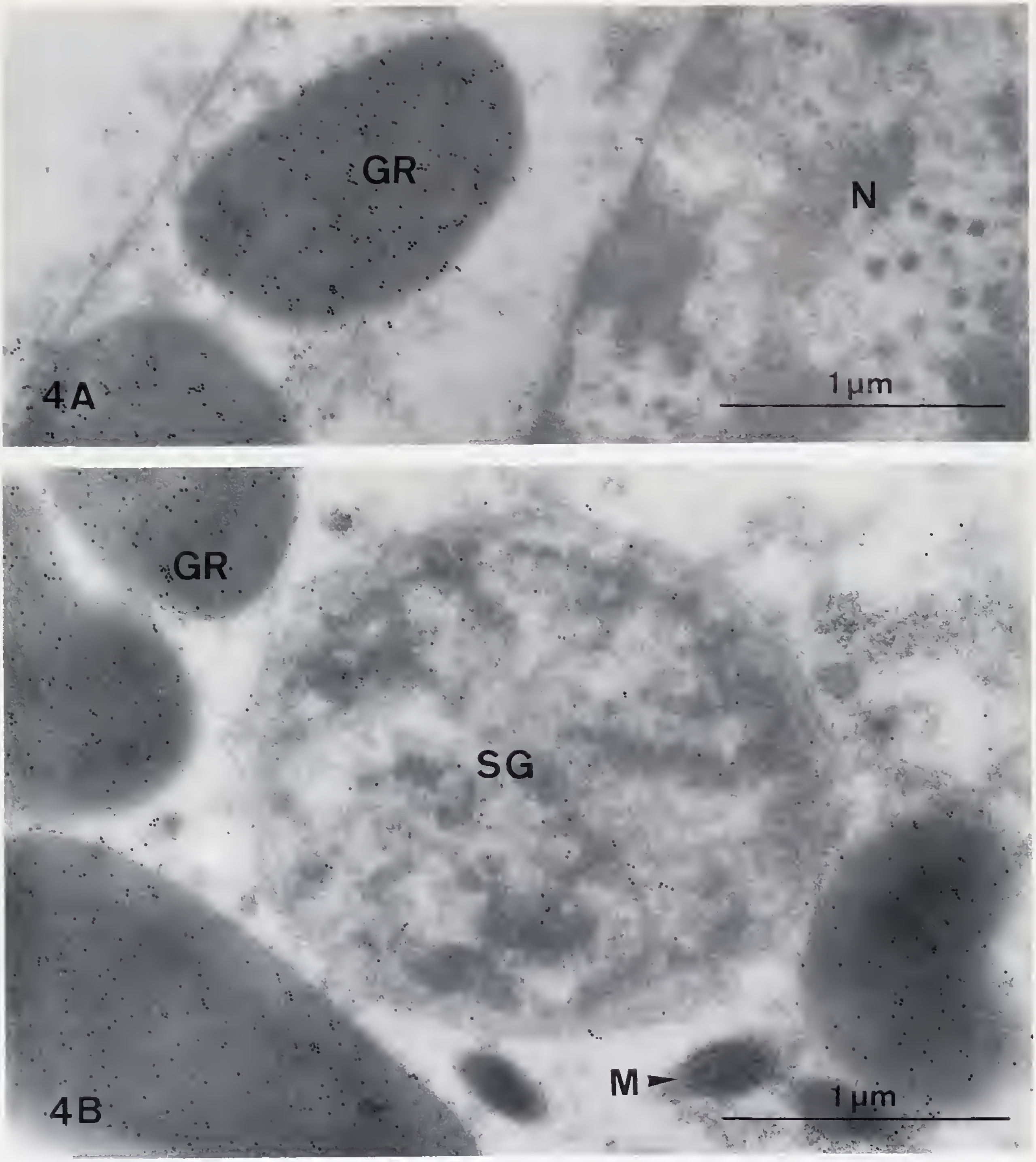
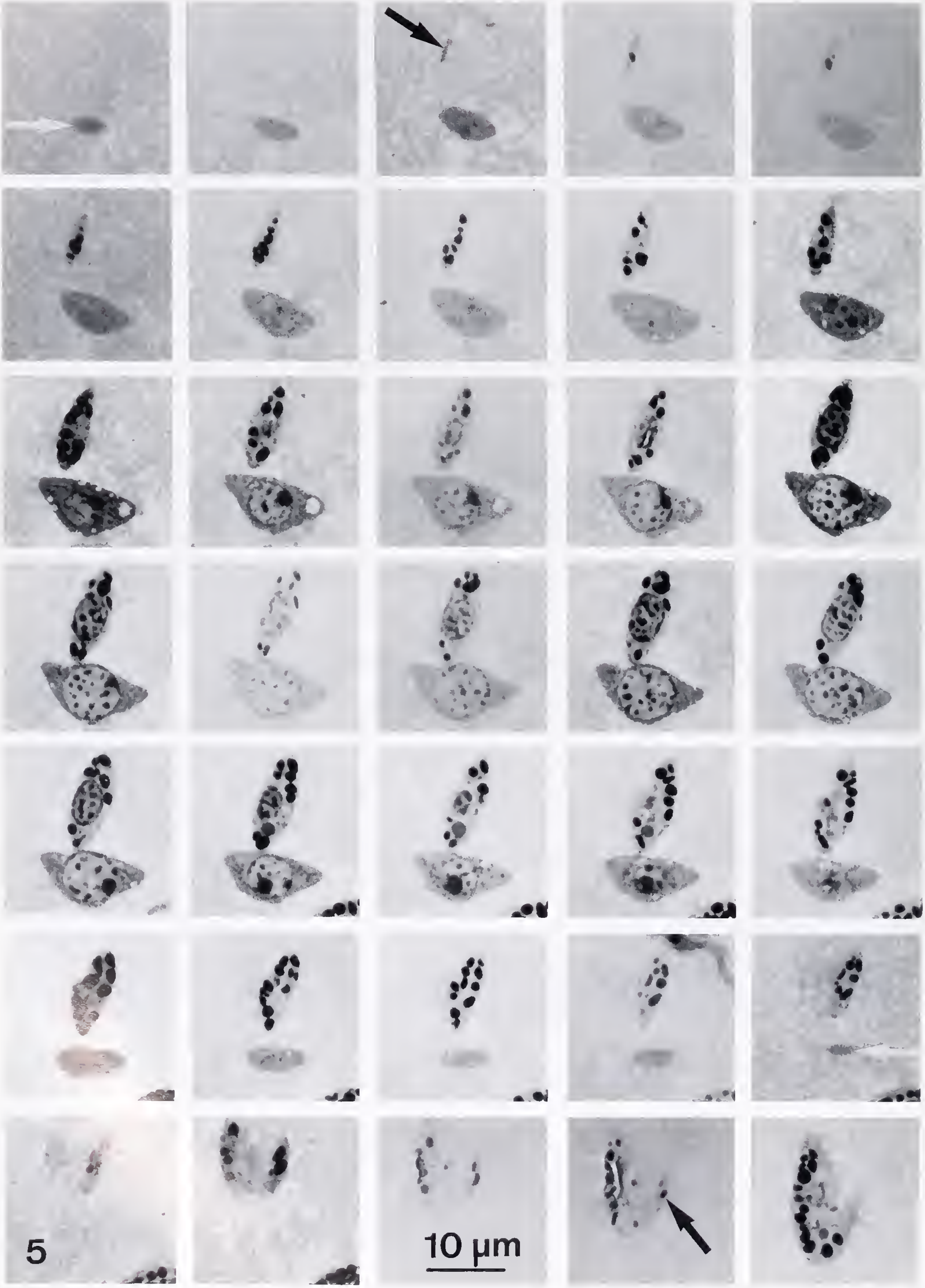


Figure 4. *Limulus polyphemus* granulocyte embedded in Lowicryl and incubated with monoclonal antibodies against coagulogen followed by gold-coupled secondary antibodies. Uniform secretory granules (GR) and structured granules (SG) are labelled. Mitochondria (M); nucleus (N).

Granulocytes

Granulocytes comprised about 99% of the hemocytes. They had a heterochromatic nucleus, a distended but poorly developed RER, few free ribosomes, few mitochondria, but many large secretory granules (Figs. 1–3, and 5). The content of the majority of the large granules is uniform, but structured


5
10 µm

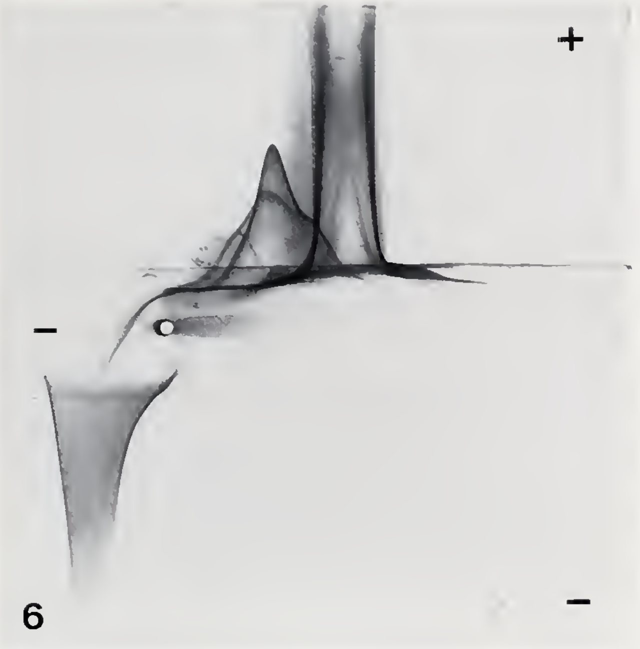


Figure 6. Crossed immunoelectrophoresis of cell-free hemolymph from *Limulus polyphemus.* The second dimension contained polyspecific rabbit antibodies against cell-free hemolymph.

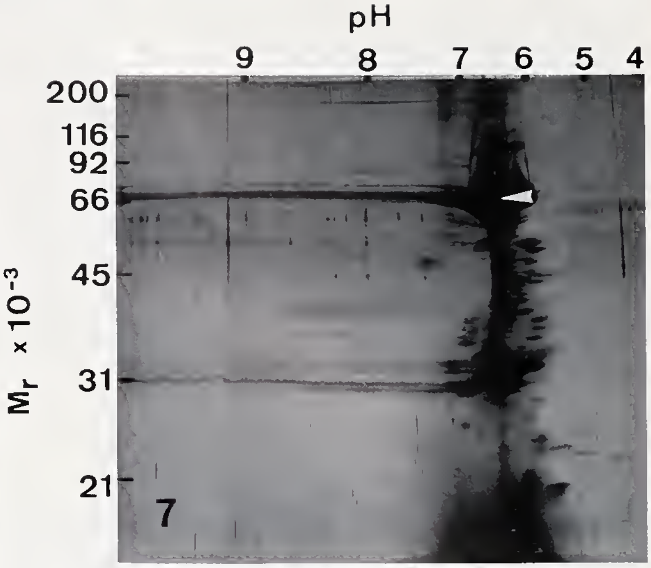


Figure 7. Cell-free hemolymph from *Limulus polyphemus* separated by O'Farrell gel electrophoresis and silver stained. Hemocyanin (arrow head).

granules are also present (Fig. 4; Dumont *et al.*, 1966; Copeland and Levin, 1985).

Plasmatocytes

Plasmatocytes were observed in all 26 *Limulus polyphemus* examined whether young or old, male or female, immediately after shipment or following more than one year in captivity. Plasmatocytes had a large, euchromatic nucleus surrounded by flattened or tubular cisternae of RER, many free ribosomes, many mitochondria, and some digestive vacuoles (Figs. 1–3). Complete serial sections of four plasmatocytes were examined by light microscopy (Fig. 5). The total number of large secretory granules observed in these cells were zero, zero, two, and two, respectively. In contrast, serial sections of hundreds of granulocytes did not reveal sections with the characteristics of a plasmatocyte (Fig. 5).

The abundance of mitochondria

The total numbers of mitochondria were counted in three independent sets of serial sections each including one granulocyte and one plasmatocyte (Table I). Plasmatocytes contain approximately three times as many mitochondria as granulocytes.

Localization of coagulogen

To reveal the ultrastructural location of coagulogen, Lowicryl embedded hemocytes were incubated with monoclonal antibodies against coagulogen followed by gold-coupled secondary antibodies (Fig. 4). In granulocytes, gold particles crowded all over the uniform secretory granules, while the number of gold particles increased with the amounts of material present in the structured granules. The density of gold particles seen in areas outside the granules is low and equivalent to background. In contrast, all 10 examined plasmatocytes with a large granule did not bind immuno-gold particles.

Characterization of polypeptides from cell-free hemolymph

The *L. polyphemus* hemolymph polypeptides immunogenic in rabbits were revealed by crossed immunoelectrophoresis (Fig. 6): the 14 polypeptides distribute themselves in 3 classes with intermediate-, low-, and cathodic mobility, respectively. The intermediate mobility group is dominating in amounts and consists mainly of hemocyanin (data not shown). The cathodic migrating group

Figure 5. Light microscopy serial sections of a plasmatocyte (white arrows) and a granulocyte (black arrows). Arrows indicate the first and last section of each cell.

is not contaminating coagulogen (Baek, 1983; Baek *et al.*, 1985).

To obtain an alternative estimate of the total number of polypeptides present in the hemolymph of *L. polyphemus,* cell-free hemolymph was separated on O'Farrell gels and visualized by silver staining (Fig. 7). Among the more than 70 polypeptides detected, the most abundant (hemocyanin monomers) had a pI around 6.3 and an Mr about 60 × 10^3, and at least 60 others had pI-values between 7.5 and 5.5. The hemolymph polypeptides range in size from Mr-values larger than 200 × 10^3 to smaller than 21 × 10^3.

Discussion

Granulocytes and plasmatocytes

We observed two kinds of circulating spheroid hemocytes in *L. polyphemus* with many and very few, if any, secretory granules, respectively (Figs. 1–3, and 5). Their main differences are summarized in Table II. The granular cells (amebocytes) are named granulocytes, while the almost agranular cells are named plasmatocytes in agreement with the terminology for other chelicerata and for insects (Gupta, 1979; Sherman, 1981; Gupta, 1985).

The granulocyte constitutes about 99% of the cells in the present study and has previously been characterized at the TEM level (Dumont *et al.*, 1966; Murer *et al.*, 1975; Nemhauser *et al.*, 1980; Ornberg and Reese, 1981; Armstrong, 1985; Copeland and Levin, 1985).

The plasmatocyte has hitherto been overlooked in *L. polyphemus* (Sherman, 1981). Its morphology is similar to the plasmatocyte recently discovered in *T. tridentatus* by LM serial sections and TEM (P. P. Jakobsen, and P. Suhr-Jessen, unpub.). Plasmatocytes are neither granulocytes having exocytosed during sampling nor cyanoblasts or cyanocytes. Plasmatocytes have the smooth ellipsoidal shape with a marginal band characteristic of the unstimulated granulocyte in contrast to the pseudopodial form following exocytosis (Dumont *et al.*, 1966; Armstrong, 1980; Armstrong and Rickles, 1982; Armstrong, 1985). Plasmatocytes all have the same size, are present in the general circulation of all animals studied at all times, and we have been unable to detect the cytoplasmic crystals normally seen in cyanoblasts and cyanocytes (Fahrenbach, 1970; Sherman, 1981). Immunogold labellings revealed coagulogen in granulocytes but not in plasmatocytes, suggesting that they have different cell lineages. This suggestion is supported by the higher number of mitochondria and free ribosomes in plasmatocytes than in granulocytes, and by morphological differences in RER and the nucleus (Figs. 2, 3). However, plasmatocytes may be granulocytes recovering from spontaneous exocytosis so early prior to harvest of the hemolymph that the marginal band of microtubules have reformed. Since the production of granulocytes in *L. polyphemus* is not continuous (Cohen, 1985), this latter interpretation implies either (1) that approximately 1% of the hemocytes in all horseshoe crabs examined in the present study are constantly recovering from spontaneous exocytosis, and that the transition from plasmatocyte to granulocyte is so fast that the frequency of intermediate hemocytes is at least one order of magnitude lower than that of plasmatocytes, or (2) that approximately 1% of the hemocytes in each animal recover from a single burst of exocytosis long before the first sampling and that this recycling is blocked at the plasmatocyte stage. It is presently unknown if the smaller fraction of plasmatocytes seen in the present study [1% compared to 3% in *T. tridentatus* (P. P. Jakobsen and P. Suhr-Jessen, unpub.)] reflects species specific differences.

Large secretory granules

Monoclonal antibodies detected coagulogen in the uniform granules and to a lesser extent in structured granules of granulocytes (Fig. 4). Together with the finding that structured granules almost always were found in close proximity to Golgi complexes, this suggests that the structured granule is an immature stage of the uniform secretory granule as suggested by Copeland and Levin (1985).

Hemolymph polypeptides

Hemocyanin is the most abundant polypeptide in the hemolymph of horseshoe crabs (Fernandez-Moran *et al.*, 1966; Armstrong 1985). In agreement with previous studies (Bancroft *et al.*, 1966), we observe that the monomers have a pI around 6.3 and an Mr around 60 × 10^3 (Fig. 7). After more than 1 year of immunizations, 14 hemolymph polypeptides are immunogenic in rabbit. However, the total number of hemolymph polypeptides is several fold larger (Fig. 6). This agrees with observations from the blue mussel and from humans (Anderson and Anderson, 1977; Suhr-Jessen and Rasmussen, 1988).

The immune defense system

Hemocytes and hemolymph polypeptides constitute the immune defense system in horseshoe crabs (Ratcliffe *et al.*, 1985). We have revealed a new hemocyte, the plasmatocyte, and identified a hitherto unforeseen high number of polypeptides in the hemolymph of *L. polyphemus.* It is tempting to speculate that granulocytes and plasmatocytes operate together, and with the hemolymph polypeptides, to recognize and destroy invading microorganisms. The techniques used in this study allow us to identify qualitative and quantitative changes in the hemolymph during and following recovery from infections, and in combination with immunoblotting to determine the specificity of the different polypeptides. Such studies may not only shed light on host-parasite interactions in horseshoe crabs and in invertebrates in general, but may also lead to improvements of the *Limulus* amebocyte lysate.

Acknowledgments

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A Viral Disease of the Ivory Barnacle, *Balanus eburneus*, Gould (Crustacea, Cirripedia)

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Abstract. The known diseases of the Class Cirripedia are reviewed. A previously unreported viral disease of the ivory barnacle, *Balanus eburneus* is described. The results of light and electron microscopic examinations of viral-infected tissues of an ivory barnacle are reported. The pathologic alterations of the parenchymal tissues and the cellular lesions produced by the invasion and replication of the virus in parenchymal cells are described. The large mature enveloped icosahedral virion (mean length 222 nm and width 175 nm) conforms to the larger viruses of the *Iridovirus* group of the family Iridoviridae.

Introduction

The class Cirripedia, the only sessile group of crustaceans, includes many diverse marine animals. The most familiar Cirripedes are the barnacles, approximately two-thirds of which are free-living and economically important as fouling agents on ships, piers, and other submerged marine structures. Barnacles are also commensals on external surfaces of whales, turtles, fish, and other crustacea and marine animals. As foreign bodies on the surfaces of these animals, barnacles may interfere with normal physiological functions and produce traumatic injuries.

Several groups of barnacles are important parasites of marine animals, such as the Rhizocephala parasitizing decapod crustaceans. Some larger species are used for human food. In spite of their importance and the need for biological control of their adverse effects, little is known about diseases that affect the Cirripedes.

Naturally occurring diseases of Cirripedes are likely often overlooked due to the difficulties of observing signs of disease in a sessile animal covered by thick calcareous plates or existing in parasitic forms hidden within a host. Accordingly, descriptions of epizootic diseases or mass mortalities of Cirripedes have been rarely reported.

No bacterial or viral diseases of Cirripedes have been previously reported. Predators, commensals, and some parasites of Cirripedes have been described (Arvy and Nigrelli, 1969; Ching, 1978; Irwin and Irwin, 1980; Williams *et al.*, 1981). Epizootics, resulting in mass mortalities, have been observed periodically in marine laboratory barnacle populations maintained for biomedical research during a eight-year period (1981–1988) in Woods Hole, Massachusetts.

Previous work by Koulish, who conducted ultrastructural studies of the ivory barnacle, *Balanus eburneus* (Koulish, 1976; Koulish and Klepal, 1981; Barden and Koulish, 1983; Koulish and Gould, 1983; Koulish and Kramer, 1986), led to the research reported here, the description of the first known viral disease of the Cirripedia.

Materials and Methods

Ivory barnacles, measuring from 1 to 2 cm at their base, were obtained from the Marine Resources Center of the Marine Biological Laboratory in Woods Hole, Massachusetts, during May, 1986. No significant mortalities were noted in the laboratory's barnacle colonies at the time the barnacles were procured, and the animals appeared grossly normal. The barnacles were maintained in the laboratory in aerated artificial seawater at 20°C and fed periodically with invertebrate marine food (principally *Artemia nauplii*).

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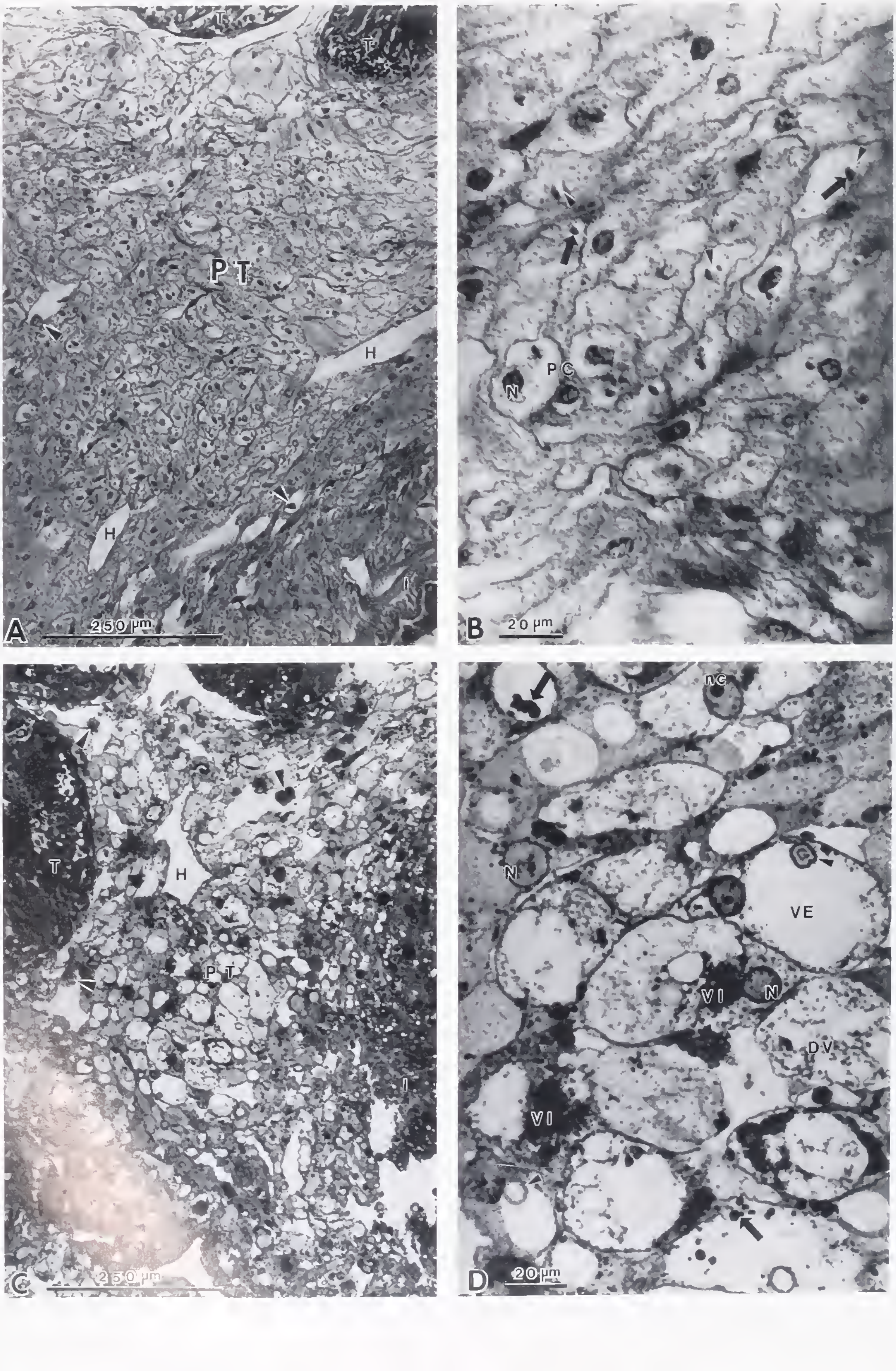
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Barnacles selected for study were rapidly removed from their shells, cut into pieces (1–2 mm in the longest axis) on a cold plate and fixed for 3 h at 4°C in a 2.5% glutaraldehyde solution buffered to pH 7.2–7.4 with 0.1 *M* cacodylate containing 3% sucrose (Koulish and Kramer, 1986). Tissues were washed briefly in 0.2 *M* cacodylate buffer then post-fixed with 1% OsO_4 in the 0.1 *M* cacodylate buffer for 1 h, stained "en bloc" with 0.5% uranyl acetate (pH 3.9) for 30 min (Terzakis, 1968), washed in several changes of 0.2 *M* buffer then dehydrated in a graded series of alcohols, and embedded in Epon 812. Thick sections (1–1 μm) were stained with toluidine blue and examined by light microscopy.

Thin sections (80–100 nm) were collected on uncoated grids and stained with saturated aqueous uranyl acetate followed by lead citrate (Koulish and Gould, 1983). Observations were made at 75 kV with a Hitachi HU 11E electron microscope.

Among the specimens of *B. eburneus* examined, the parenchyma of one barnacle was found to be heavily infected with virus-like particles. The pathological alterations of the viral-infected parenchymatous tissues and cells and the details of viral replication were examined by light and transmission electron microscopy, photographed, recorded, and described.

Electron micrograph magnifications and measurements of viral particles were calibrated at three different magnifications by means of germanium-shadowed carbon replicas of a ruled diffraction grating, with a distance of 882 nm between the lines (Agar, 1967).

Results

Light microscopic examination

The "parenchymatous tissues" described by Tornava (1948) and Koulish (1976), surrounded and supported the visceral organs such as the gonads, ducts, and intestines in the mantle cavity. The hemolymph tissue spaces and vessels, which frequently contained hemocytes, traversed the parenchymal tissues (Fig. 1A). The normal parenchymal cells were pleomorphic interlocking cells that contained a variety of electron-dense and lucent cytoplasmic granules (Fig. 1B). In spite of their variable shapes, normal parenchymal cells were relatively uniform in size, except in close proximity to outer walls of visceral organs, where they formed a more dense adventitial coat of parallel compact cells. The latter cells were smaller, more elongated, and more darkly stained (Fig. 1A).

A "special" morphologically differentiated type of parenchymal cell containing many highly refractile zinc phosphate granules underlies the midgut region of barnacles and has been described by Koulish (1976).

In the virus-infected barnacles, histopathological lesions were limited to the parenchymal tissues. Cytopathic alterations of parenchymal cells were characterized by progressive disruption of cell structures (Fig. 1C). The nuclei showed margination and lysis of chromatin and hypertrophy of the nucleoli. The plasma membranes became distorted and poorly defined. Within the cytoplasm, dense granular collections and inclusion bodies were surrounded by lysed areas. The cytoplasmic granules, found normally in parenchymal cells, were frequently distorted, enlarged and enclosed in large vacuoles. Cellular edema and hypertrophy were pronounced. Inclusion bodies appeared to fragment and release their contents into the cleared cytoplasmic spaces and vacuoles. The nuclei of some infected cells were difficult to observe in the greatly swollen degenerated cells (Fig. 1D). Similar destructive changes were observed in individual hemocytes contained in hemolymph vessels and spaces within the parenchymatous tissues.

The architecture of the parenchymatous tissue became altered due to swelling, dissociation, displacement, and rupture of individual cells. Abnormal tissue spaces and irregularly shaped cords of cells formed (Fig. 1C, D). The adventitial parenchymatous tissues surrounding the vis-

Figure 1. Photomicrographs of parenchymal tissues and cells of uninfected and virus-infected ivory barnacles:

(A) Note the relatively uniform interlocking appearance of the uninfected parenchymal tissue (PT), the more elongated and compressed adventitial coat of parenchymal cells and testis (T), and the hemolymph spaces (H) containing hemocytes (arrows).

(B) Note the relatively uniform uninfected interlocking parenchymal cells (PC) and their nuclei (N), and the dark (large arrows) and light (small arrows) cytoplasmic granules.

(C) Note the disrupted architecture of the virus-infected parenchymatous tissue (PT), the irregularly shaped swollen vacuolated parenchymal cells, distended hemolymph spaces (H), containing swollen hemocytes (arrows), and the dark-staining adventitial cells containing many small refractile granules surrounding the intestine (I).

(D) Note the greatly enlarged virus-infected parenchymal cells with enlarged nuclei (N) and the nucleoli (nc), irregularly shaped cytoplasmic viral inclusion bodies (VI) and dispersed virions (DV), swollen light-staining granules (small arrows) and dark granules (large arrows).

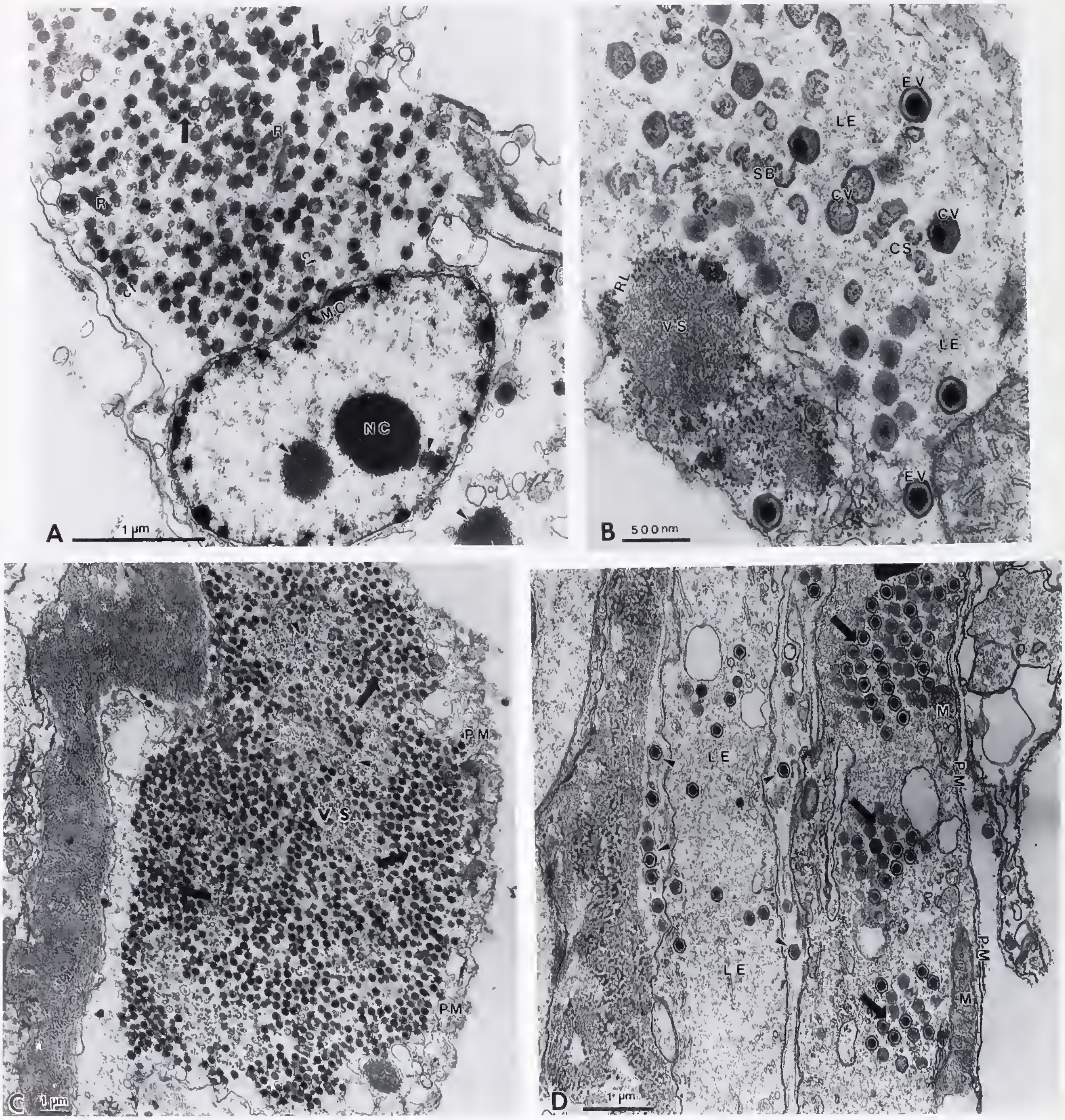
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Figure 2. Electron micrographs of virus-infected parenchymal cells:

(A) Note the marginated chromatic (MC), the enlarged nucleolus (NC) with finely granulated loosely attached fragments within nucleus and cytoplasm (small arrows). Note the viral assembly in the cytoplasm, including rod-like forms (R), condensing-forms (cf), and non-enveloped virions (large arrows).

(B) Note the virogenic stroma (VS), lysed endoplasmic reticulum (LE), ribosomal-like particles (RL) assembled at the periphery of the virogenic stroma, crescent-shaped strands (CS), spiral-shaped brands (SB), condensing immature non-enveloped virions (CV), mature enveloped virions (EV), and mitochondrium (lower right).

ceral organs were less altered in shape but contained numerous highly refractile small colorless granules.

Transmission electron microscopic examination

In addition to the histopathologic changes observed by light microscopy, ultrastructural details of pathologic alterations of virus-infected cells and viral morphogenesis were obtained by transmission electron microscopy.

The light microscopic observations of nuclear hypertrophy, chromatin margination and lysis, and nucleolar hypertrophy were confirmed. In addition, nucleolar-like structures appeared to break up into finely granulated loosely aggregated fragments that were released within the nuclear and cytoplasmic compartments (Fig. 2A).

Viral replication and morphogenesis occurred at the periphery of the irregularly shaped granular electron-dense cytoplasmic inclusion bodies (virogenic stroma) in the cytoplasm of the parenchymal cells. Ribosome-like particles associated with the partially lysed endoplasmic reticulum were observed at the surface of the virogenic stroma (Fig. 2B). Virionic components appeared to assemble along the periphery of the stroma.

Based upon the apparent stages of development, viral morphogenesis began at the periphery of the viroplasm with the formation of fine filaments and the assembly of small crescent-shaped bilaminated strands within the disrupted endoplasmic reticulum (Fig. 2B).

The strands appeared to fuse and increase in length, forming long spirals that coalesced into less electron-dense oval or hexagonal outlined bodies, ranging approximately from 115 to 192 nm in length, and 92 to 154 nm in width. As the assembly process continued, the rounded bodies could be recognized as incomplete viral particles undergoing further assembly (Fig. 2B).

Occasionally, membrane-bound stout rod-like structures were observed in assembly areas (Fig. 2A).

The virionic assembly appeared to continue as the core or nucleus of the particle became more electron-dense and surrounded by a capsid. The viral assembly process extended throughout the mass of viroplasm, where all stages of assembly could be observed simultaneously. However, the greatest number of mature enveloped virions were found at the periphery of the vironic mass (Fig. 2C). With the exception of the adventitial parenchymal cells, virions were more widely dispersed within the cytoplasm of parenchymal cells evidencing marked degeneration and necrosis.

Upon completion of the viral assembly process in adventitial parenchymal cells, the mature virions were arranged in small arrays within the cytoplasm of the more elongated and compressed cells and could also be found in the intercellular spaces (Fig. 2D).

The distribution of these small viral arrays corresponded to the deeply staining granules seen in the adventitial parenchymal cells at the light microscope level.

The nucleocapsid ranged from approximately 134 to 154 nm in length, and 106 to 110 μm in width. The mature enveloped virions had a calculated length ranging from 184 to 253 nm, with a mean of 222 nm; and a width ranging from 147 to 216 nm, with a mean of 175 nm. The viral envelope, consisting of an inner matrix, a middle lipid bilayer, and an outer layer of peplomers could be seen surrounding the mature virions (Fig. 3).

Discussion

The disease is the first reported viral infection of barnacles or of any other member of the class Cirripedia. The viral agent had a predilection for parenchymatous cells of the barnacle, invading and replicating within these cells. Hypertrophy, degeneration, and necrosis of the parenchymatous cells and tissue resulted from the infection.

Although these cells have been designated as parenchymal cells, their exact function remains unknown. The term "parenchymal cells" has been reserved for the distinguishing or specific tissue cells of a gland or organ, as opposed to cells which form the supporting structure or stroma (Taylor, 1946).

While the parenchymal cells fill the spaces between the organs in the mantle cavity of barnacles, their function appears to be more than those of connective tissue. The relationship of parenchymal to hematopoietic tissues in barnacles has been a subject of speculation (Walley, 1969). The relationship of hemocytes to parenchymal cells is further suggested by the detection of viral-infected hemocytes in this study. Although some of the hemocytes were infected, the importance of hemolymph infection during the course of the disease was not established. The specialized (morphologically differentiated) types of parenchymal cells, indicated in this and other studies,

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(C) Virogenic stroma (inclusion body) (VS) in cytoplasm of an adventitial parenchymal cell. Note the concentration of mature virions at the periphery of the stroma (large arrows) and immature virions in the process of assembly within, and the degenerating plasma membrane (PM).

(D) Note the small viral arrays (large arrows) within the cytoplasm of adventitial parenchymal cells, virions dispersed in the lysed endoplasmic reticulum (LE) and intercellular spaces (small arrows), the degenerating plasma membrane and adjacent mitochondria.

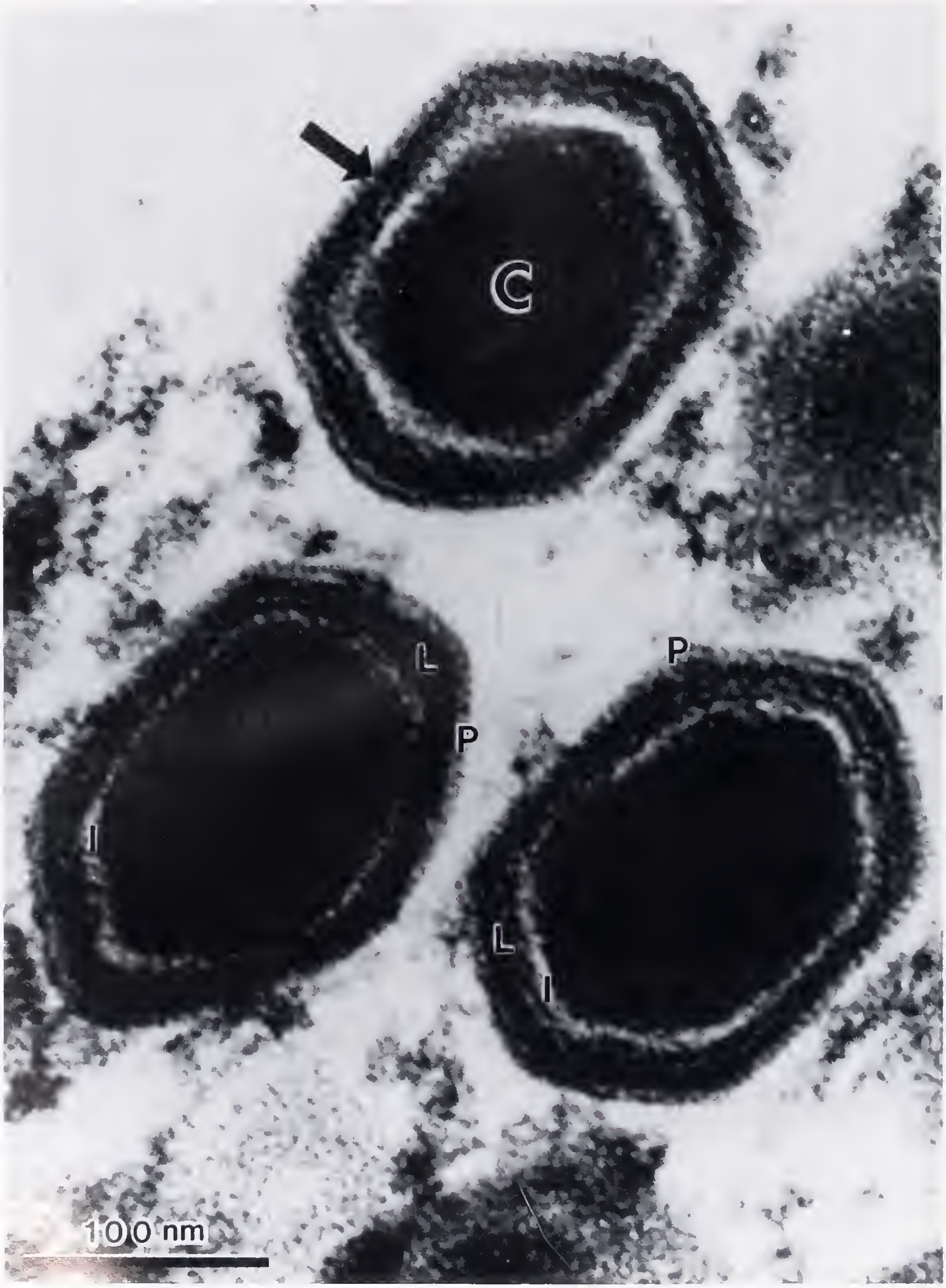

Figure 3. Mature hexagonal (icosahedral) enveloped virions (arrow) within the cytoplasmic space of infected parenchymal cell. Note the nucleocapsid core (C) and its capsomeres, the well defined envelope, consisting of an outer layer of peplomers (P), a middle lipid bilayer (L), and an inner matrix layer (I).

suggest a variety of functions for these cells, which require further studies.

The morphological characteristics of the virion, their dimensions, and the method of viral replication and assembly, in close association with the cell's plasma membrane and mitochondria, conform to those reported for the family Iridoviridae, the largest of the cytoplasmic icosahedral DNA viruses (Hess, 1981; Fraenkel-Conrat and Kimball, 1982; Willis, 1985; and Fenner *et al.*, 1987).

Of the known iridovirus infections of arthropods, infections have been frequently reported in insects (Kelly and Robertson, 1973; Carey *et al.*, 1978); and in crustaceans, including isopods (Federici, 1980), and branchiopods (Frederici and Hazard, 1975).

Iridovirus infections have also been reported in mammals (Fenner *et al.*, 1987).

More studies are needed to determine the epizootiology, incidence, pathogenesis, comparative pathology, and the relative importance of this viral disease of barnacles and other members of the class Cirripedia.

Acknowledgments

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Spectral Sensitivity of Vision in the Mantis Shrimp, *Gonodactylus oerstedii,* Determined Using Noninvasive Optical Techniques

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Abstract. Compound eyes of stomatopod crustaceans have many unique design features. Recently developed intracellular optical physiology techniques permit the measurement of spectral sensitivity functions in intact eyes of these animals. We tested four technically distinct approaches to measurement of spectral sensitivity in peripheral ommatidia of the compound eyes of *Gonodactylus oerstedii.* Each technique was evaluated for (1) the time required to measure a complete sensitivity spectrum, (2) reproducibility of results, and (3) suitability for use in a fully automated system. A spectral scan technique, in which the visual response is held constant by varying stimulation intensity throughout the scan, was found to be superior. With it, a complete, highly reproducible measurement of spectral sensitivity from 400 to 650 nm at 10-nm intervals could be accomplished automatically within 20 min. The photoreceptors were maximally sensitive near 540 nm, and the sensitivity curve was well described by the absorptance curve for a retinal$_1$-based visual pigment with peak absorbance at 537 nm and peak density equal to 0.5 OD.

Introduction

The compound eyes of stomatopod crustaceans are among the most unusual visual organs ever evolved, and several laboratories have recently taken up their investigation. These new studies confirm Exner's (1891) original observation that vision in these animals is triple; as many as three sets of ommatidia in a single compound eye may view the same spatial region (Horridge, 1978; Schiff *et al.,* 1985, 1986a, b; Cronin, 1986; Schiff and Candone, 1986). Each eye is divided by a central band of 2 to 6 ommatidial rows; patches of ommatidia on each side of this band share fields of view with it.

The triple design is conserved among three of the four stomatopod superfamilies (Manning *et al.,* 1984), and offers unique opportunities for color vision. For instance, in the gonodactyloid stomatopods, where the central band includes six ommatidial rows, each is of special design (Marshall, 1988). Colored filters, which must affect the spectral sensitivities of the underlying photoreceptors, are found in some of these rows. Thus, the various rows, as well as ommatidia outside the central band, may vary in spectral sensitivity; and as they simultaneously view an object its color may be analyzed (Marshall, 1988). Gonodactyloid stomatopods frequently live in shallow, well-lit habitats, and are marked with striking, species-specific colors (Caldwell and Dingle, 1975), so color vision should be not only possible, but useful.

Learning how stomatopod visual systems respond to pigmentary colors requires the measurement of spectral sensitivity in the various rows of the central band as well as in the more peripheral regions of the eye. Recently, we developed a noninvasive, optical approach to studying crustacean ocular physiology (Cronin, 1989) that is based on intracellular optical physiological techniques developed to study insect vision (*e.g.,* Franceschini, 1975; Stavenga and Kuiper, 1977; Bernard and Stavenga, 1979; Bernard and Wehner, 1980). Measurements are made of changes in reflectance that occur within the deep pseudopupil (Stavenga, 1979) of a selected region of the compound eye as it adapts to light or dark. These changes are caused, at least in insects, by the light-mediated translocation of granules of retinular cell screening pigment to the region of the rhabdom, where

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they reduce the light flux within the photoreceptor (see review of Stavenga, 1979).

Crustacean retinular cells also contain mobile pigment granules (Ludolph *et al.*, 1973; Schönenberger, 1977; Stowe, 1980), which probably bring about the intracellular optical response in their eyes (see also Cronin, 1989). However, in some insects, secondary pigment cells also may respond to light stimuli, which may confound measurements of photoreceptor cell spectral sensitivities (Hamdorf *et al.*, 1986; Land, 1987). Although the secondary response appears to occur only in superposition compound eyes, the use of the technique must be rigorously justified when it is applied to new species.

Optical techniques offer the advantages of (1) knowing precisely what ommatidial region is responding in an experiment and (2) being able to use the same eye repeatedly, so that a variety of regions can be examined. However, because the intracellular optical response is slow relative to electrophysiological responses, the time required for spectral sensitivity measurements can be lengthy. Recently, new approaches and automation have greatly increased the speed and resolution of electrophysiological measurement of spectral sensitivity (Gribakin, 1981; Franceschini, 1984; Menzel *et al.*, 1986; Paul *et al.*, 1986; Steiner *et al.*, 1987). It seemed likely that optical measurements of spectral sensitivity could similarly be improved.

Therefore, we surveyed a variety of methods of measuring spectral sensitivity in a single region of the compound eye of a gonodactyloid stomatopod, *Gonodactylus oerstedii*, using the intracellular optical approach. The selected region (the deep pseudopupil of ommatidia in the dorsomedial region of the eye) probably has only one spectral mechanism operating between 400 and 700 nm (Cronin, 1989), making it relatively easy to compare the results of each approach. The study has led to the development of a spectral scan technique permitting the measurement of a sensitivity spectrum within 20 min.

Materials and Methods

All animals were collected in the Florida Keys and shipped to Maryland for study. A detailed description of the intracellular optical physiological technique, as applied to *G. oerstedii*, is given in Cronin (1989). Here follows a brief description.

Animals were attached dorsal side down to an adjustable, submersible stage using Scutan dental plastic; their eyes were immobilized using the same material. The animal was covered with artificial seawater and adjusted so that all three pseudopupils of the ventral part of one eye were visible. Prior to each experiment, the experimental mantis shrimp was dark-adapted for at least several hours, and commonly overnight.

The deep pseudopupil of the dorsomedial region of a selected eye was centered in the field of view of an incident-light, photometric microscope. Two beams of light were brought into the incident-light path on the same axis, passed through a Zeiss 25-mm Luminar objective protected with an angled, submersible diptube, and entered the eye. One of these, the measuring beam, contained long-wavelength light (>720 nm; Schott RG720 longpass filter) and remained illuminated throughout the experiment. A portion of this beam returned to the photometric microscope after being reflected and/or scattered by material within the deep pseudopupil. The intensity of the returning light alters with dark or light adaptation; these changes provide the signal for measuring visual responses (Cronin, 1989). This intensity was measured with a photomultiplier (Hamamatsu R928) connected via a PARC 1140C quantum photometer to the A/D interface of a microcomputer. Light arriving at the photomultiplier passed through a 720-nm longpass filter, which permitted passage of the measuring beam alone. In these experiments, the measured area of the eye was restricted to about 10 ommatidia by an adjustable rectangular diaphragm mounted in the microscope's upper image plane.

The other beam, the stimulating beam, reached the eye only during the course of a stimulation. Its plane of polarization was set parallel to the line connecting the three pseudopupils of the eye under study, while its wavelength, intensity, and duration could be controlled with the assistance of the microcomputer. Wavelength was altered using an Oriel model 7240 grating monochromator, intensity was adjusted with counterrotating 10-cm diameter quartz neutral density wedges (total density range, 0–3.8), and exposure duration was set using a Uniblitz electromagnetic shutter. The stimulating beam was produced by a Xenon arc lamp, either 75 W or 150 W; its quantal irradiance at each test wavelength was measured daily using a calibrated United Detector Technology PIN-10DP/SB photodiode at the position of the experimental eye. The way in which the stimulating beam was presented varied with each of the experimental approaches, as described below.

Criterion technique

Here, we determined the intensity of light required at each wavelength to elicit a criterion response. Wavelengths were tested every 20 nm from 400 to 660 nm, presented in random order. The response was taken as the average reflectance level during the final 2 s of a 10-s stimulus (see Fig. 1), compared to the average level of reflectance in the dark. The criterion response level was set at 2 to 3%, depending on the responsiveness of the eye under study. Stimulations occurred at 1.5-min intervals. To correct for possible drifts in sensitivity during an experiment, all sensitivities were compared to that at 500 nm, and the 500-nm standard sensitivity was measured frequently throughout each run.

Interpolation technique

Ten-second stimulations were provided at single wavelengths at 1.5-min intervals at various intensities. Responses were quantified as in the criterion experiment, and the experimenter selected intensities expected to produce responses of about 1%, 2%, 4%, and 8%. Data were plotted as percent response *versus* log quantal intensity, and the intensity required for a standard response of 5% was determined from a linear regression line fitted to the points. As in the criterion experiments, sensitivities were compared to that at 500 nm, which was frequently redetermined. Wavelengths at 10-nm intervals from 400 to 640 nm were presented in random order.

Ramp technique

In these experiments, eyes of experimental animals were given a slowly increasing stimulation intensity at each wavelength, and the intensity required for a criterion response was determined by interpolation. The neutral density wedges were set so that the initial stimulation occurred at a level where no response was measureable. Responses were averaged into bins 2 s in length. Each stimulation sequence began with a 20-s dark period. The shutter opened and measurement continued for 10 s longer. The neutral density wedges were then rotated stepwise to decreasing densities, with each step (a density change of about −0.03) following a 2-s measurement period. The intensity continued to increase until the reflectance was 5% above the level measured during the initial 20-s dark period, at which time the shutter closed and the wedges rewound to their initial positions. Subsequent ramps were presented after a 2.5-min dark period. The response curve was smoothed by averaging over three adjacent bins, and the intensity required for a 4% response level was computed from the smoothed curve. As for criterion and interpolation techniques, measured sensitivities were compared to that at 500 nm, which was frequently redetermined. Other wavelengths were presented in random order.

Scan technique

In these experiments, a negative-feedback cycle kept the response "clamped" at a particular level; stimulation intensity was varied at each successive wavelength to maintain the constant response level. Scans began with a 50-s dark period for measurement of the dark-adapted reflectance level. The stimulation wavelength was then set to 400 nm, the intensity set to a level previously determined to produce a response of about 10%, and the animal was exposed to light for 20 min to attain a stable light-adapted reflectance level. A 50-s measurement of this reference reflectance level was then taken. The wavelength was then changed to a new value; in an ascending scan this would be 410 nm, while in a descending scan it would be 650 nm. Measurements were taken in 5-s bins. The average reflectance in each bin was compared to the light-adapted reference reflectance. If the two values did not match to within 0.25%, the density wedges were rotated so as to drive the response towards the desired reference. Thus, if reflectance exceeded the reference level, the density was increased (reducing stimulation intensity), and *vice versa.* Once the match occurred, the current wavelength and quantal intensity were stored, and the monochromator was set to the next wavelength, 10 nm above (ascending scan) or below (descending scan) the current wavelength. Ascending scans continued to 650 nm, and descending scans to 410 nm. When successive scans were taken, the reference level to 400-nm stimulation was once again measured without an intervening stabilization period. Successive scans occurred in opposite directions.

Results

The properties of the intracellular optical responses of *Gonodactylus oerstedii* have been described (Cronin, 1989). Typical responses of a fully dark-adapted eye at two stimulation intensities are illustrated in Figure 1. The eye remained in the dark (only the measuring beam was illuminated) until 0 s, when stimulation commenced. Reflectance from the deep pseudopupil rose to a new steady state in 5 to 10 s. Above threshold and below saturation, the response increased monotonically with quantal intensity. When the stimulation ceased, the reflectance level returned to the dark-adapted baseline in 5 to 10 s. The response levels were computed by comparing the average level in the final 2 s of the stimulation interval with the levels before the stimulus commenced (2-s interval before 0) and during the final 2 s of the post-stimulation period. In Figure 1, these responses were 28.4% (upper trace) and 3.65% (lower trace).

Criterion experiments

The criterion level was set at 2% or 3%, which is far below the maximum response of 25 to 40%. Spectral sensitivity was derived by determining the sensitivity (the inverse of the photon flux) at each test wavelength relative to that at the standard wavelength (500 nm). Each resulting function was normalized to its respective peak, and the overall average curve and standard errors were

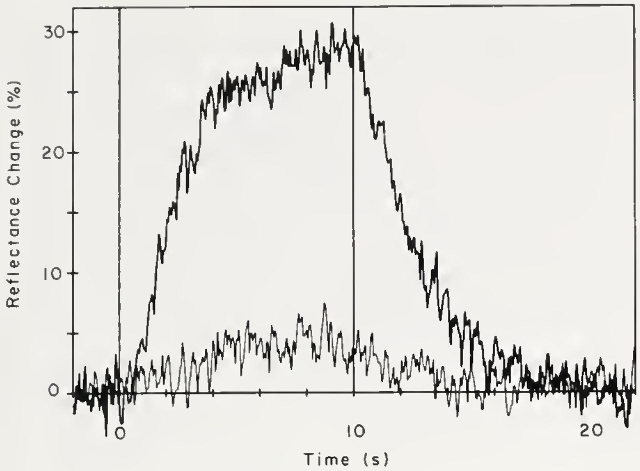

Figure 1. Typical intraocular optical responses, as changes in reflectance, in the deep pseudopupil of the medial ocular region of a compound eye of *Gonodactylus oerstedii.* Stimulation commenced at 0 s (1st vertical line) and ended 10 s later (2nd vertical line). The measuring beam, which was illuminated throughout the experiment, contained wavelengths >720 nm; light of this composition sensitizes the response (see Cronin, 1989). Quantal stimulation intensities were 1.79×10^{13} photons cm^{-2} s^{-1} (upper, dark trace) or 5.61×10^{10} photons cm^{-2} s^{-1} (lower, light trace), which caused responses of 28.4% and 3.65%, respectively.

computed. These were normalized to the peak of the average curve. The resulting function (Fig. 2) peaks at 520 nm, falls away steeply at longer wavelengths and less steeply at shorter wavelengths, and resembles a rhodopsin absorption spectrum. Finding the precise intensity for a criterion response often required several stimuli, so about 3 h were required to measure a single function. The other experimental approaches were designed both to reduce this time and to automate the procedure as much as possible.

Interpolation experiments

In these, the response to an initial stimulation was noted, and subsequent stimulation intensities were selected to produce responses ranging from 1 to 8%. Since the response was compared to both the initial and final dark-adapted levels, two values were obtained at each intensity. Regression lines were computed from the eight resulting points; typical response *versus* log intensity curves are plotted in Figure 3A. The photon flux required for a 5% response at each wavelength was compared to that required for an equivalent response at 500 nm, and sensitivity functions were computed as in the criterion experiments. The average curve for three such experiments (Fig. 3B) has a maximum at 530 nm, and its shape is similar to the function of Figure 2. An entire spectral-sensitivity determination (10-nm intervals, 400–640 nm) required some 3 to 5 h, which was somewhat longer that the time taken for a criterion run. However, more wavelengths were sampled in the interpolation experiments; both methods required similar times for the measurement of each single point.

Ramp experiments

Here we automated the collection of sensitivity spectra. Each stimulation sequence began at a subthreshold intensity and continued until a 5% response was reached. The entire run was executed under computer control, following a list of test wavelengths. A sample response is given in Figure 4A. The eye remained in the dark until the shutter opened at the time indicated by the vertical line, and the neutral density wedges began to step toward lower densities at the time indicated by the arrow. Steps occurred at 2-s intervals. The response curve (solid line) was smoothed by taking a running three-point average (the first and final points were not averaged). In each presentation, the intensity at which the smoothed curve crossed the 4% response level (the horizontal line on the graph) was determined, and sensitivity curves were computed as in the previous sections. The average curve (5 experiments, Fig. 4B) resembles the previous results (Figs. 2, 3B), but contains fewer points. Each run required some 2 h. Furthermore, if animals cleaned their eyes or waved appendages into the measuring beam, large signal fluctuations resulted that disturbed the ramp and frequently aborted measurements. In contrast, in the manual techniques the experimenter easily recognized such artifacts and excluded them from further consideration.

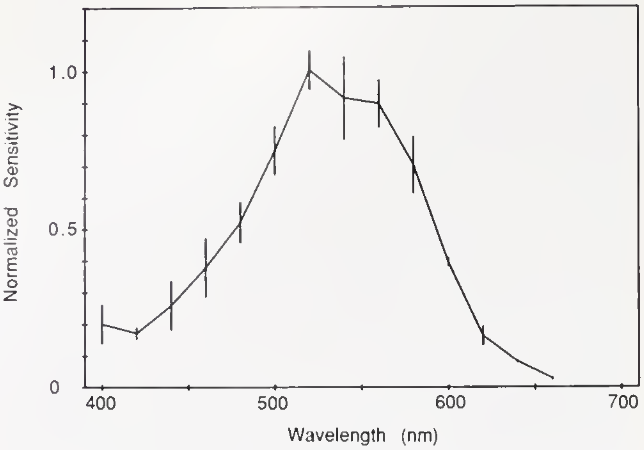

Figure 2. Criterion technique. The curve is the average of four determinations of spectral sensitivity, as described in the text. The vertical lines at each plotted point are the standard errors of the mean.

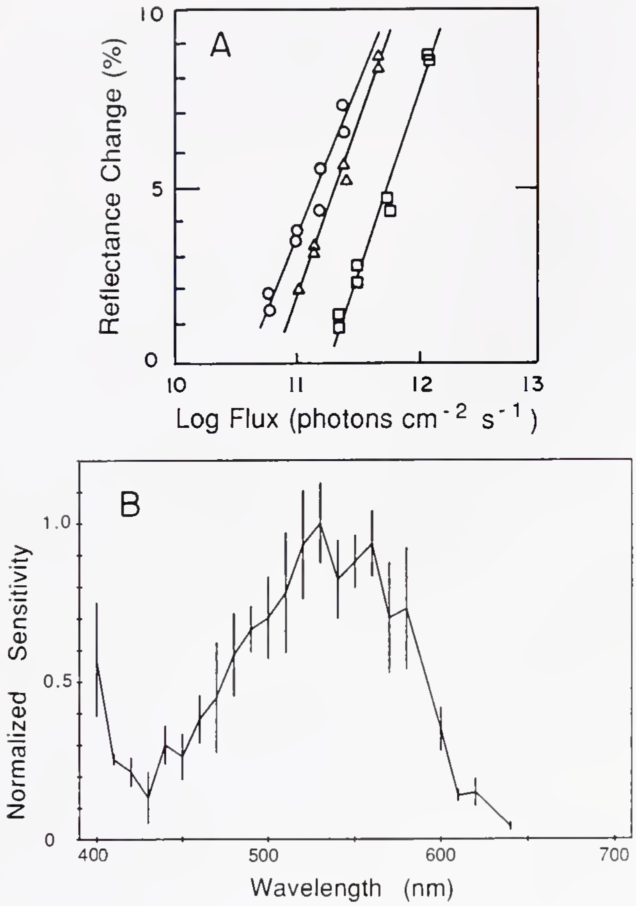

Figure 3. Interpolation technique. A. Response vs. log intensity functions at 3 wavelengths: 550 nm (circles), 500 nm (triangles), and 450 nm (squares). Each set of points is fit with a linear regression function, and the intensity necessary for a 5% response is determined from this. B. Average sensitivity measured by this technique. The curve is the average of three runs; vertical lines are standard errors of the mean.

Spectral scan experiments

This technique was inspired by electrophysiological measurements made by Franceschini (1984) and by Menzel *et al.* (1986). The underlying principle is to "light clamp" the response to any desired level by adjusting stimulation intensity. In our experiments, each measurement was taken over a 5-s interval, approximately one time constant for a typical intracellular optical response. The computer then decided whether to accept the response level or to alter the stimulation intensity and repeat the measurement. As successive measurements occur at wavelengths separated by only 10 nm, relatively little adjustment is required over most of the sensitivity band. Furthermore, any artifacts caused by animal movements are very unlikely to produce signals near the reference level, and so are automatically rejected. The reference level of the response was generally near 10%, somewhat higher than in the previous experiments. This level was chosen to allow the measurement of both negative and positive changes, and to provide some headroom for drift which might occur during a single scan.

The data of an ascending spectral scan, in which the reflectance level was clamped 12.9% above that measured in the unstimulated condition, are plotted in Figure 5A. Each time wavelength increased, a spike appeared in the record, which was subsequently eliminated as the scanning program brought the response to within 0.25% of the reference level. While the form of the record depends to some extent on the spectral distribution of the stimulating beam, at times when sensitivity is increasing

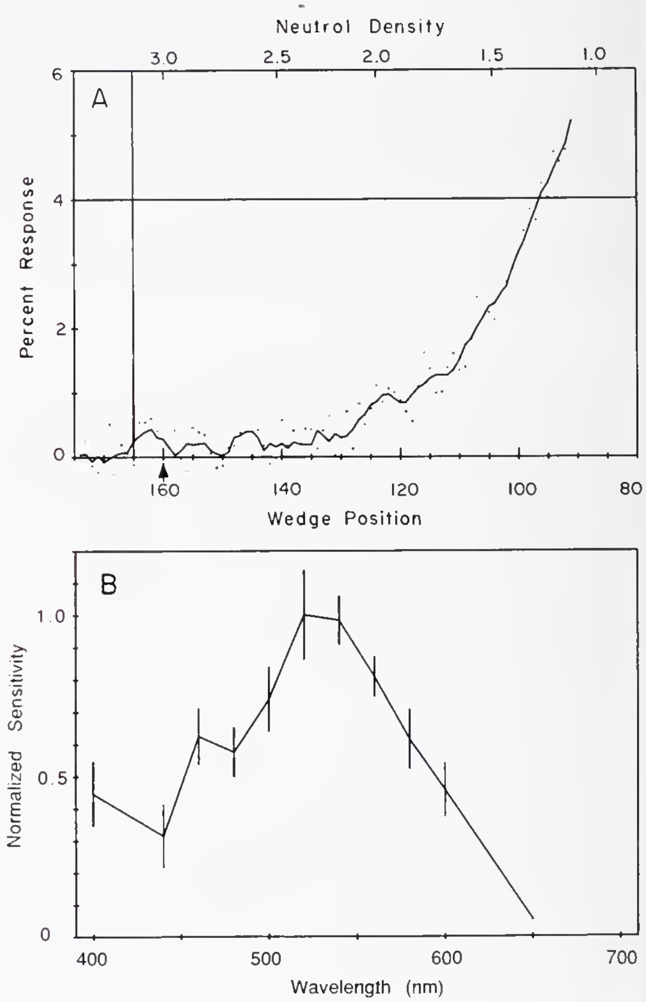

Figure 4. Ramp technique. A. Results of a single measurement (500 nm, reference intensity of 5.68 × 10^{12} photons cm^{-2} s^{-1}). The eye was initially in the dark (measuring beam only). The shutter opened at the time indicated by the vertical line, when the neutral density wedges were at position 160. At the time indicated by the arrow, the wedges began to rotate to positions of decreased density, with steps (density loss of 0.03/step) occurring at 2-s intervals. When the response reached 5%, the exposure ended. B. Average results of five experiments. Vertical lines indicate standard errors of the mean.

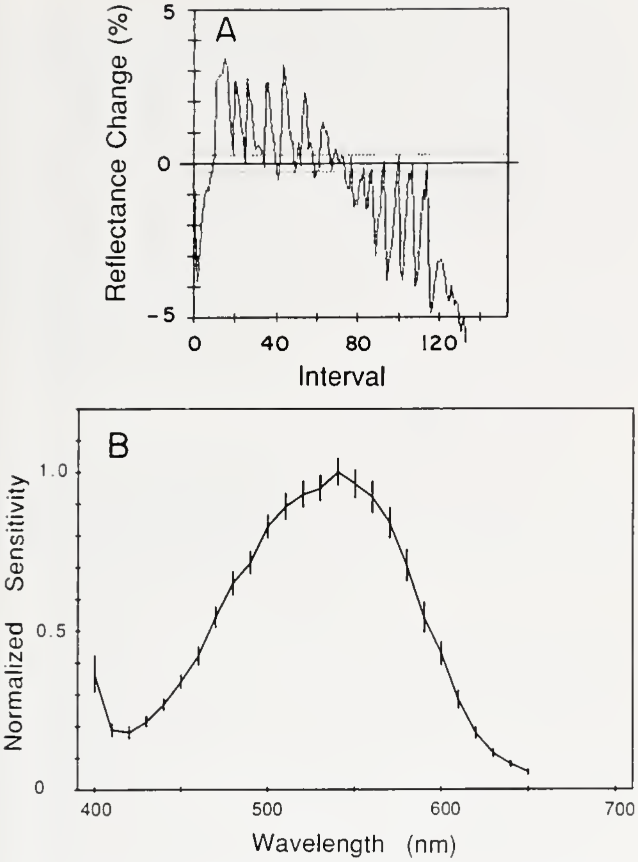

Figure 5. Scan technique. A. Results of a single ascending spectral scan. The scan began at 400 nm at interval 0, and each interval lasted 5 s. Whenever reflectance fell within 0.25% (dotted lines) of the reference level (0% reflectance change), the wavelength and photon flux was recorded and the wavelength was driven to a value 10 nm greater. Whenever the reflectance differed from the reference by more than 0.25%, the density wedges were rotated to alter the stimulation intensity and move the response towards the reference level. In general, each spike in the record occurred as a new wavelength was reached and the negative-feedback loop restored the reflectance to the reference level. The scan ended when the wavelength reached 650 nm. B. Average results of 21 scans in 16 experiments. Vertical lines indicate standard errors of the mean.

successive spikes tend to point upwards, as in the earlier part of the record. As the peak sensitivity is passed, the spikes begin to point downwards, so stimulus intensity must be increased to bring the response back to the reference level. In the illustrated scan, the longest wavelengths contained insufficient light to bring the response to the reference level, and the trace trailed off to decreasing levels of reflectance.

Sensitivity curves were analyzed and averaged as before. The average curve and associated errors for 21 separate scans, taken from 16 individual presentations (some included multiple scans), are plotted in Figure 5B. Scans typically were completed within 15 to 25 min, once the initial 20-min stabilization period was passed (the scan of Fig. 5A required 20 min, 19 s). Thus, starting with a fully dark-adapted eye, four complete scans (2 ascending and 2 descending) could be completed in 2 h or less. Though collecting the data of Figure 5B required a similar amount of time as data collection for the earlier figures, the resulting curve is smoother, and the errors smaller, than in any of the previous data sets. Individual scans tend to be less variable than the separate data sets of the other techniques as well. At moderate levels, response is nearly a linear function of log intensity (Fig. 3A), so the increased precision is unlikely to be due only to the somewhat higher levels of response chosen here.

Discussion

The primary purpose of this study was to develop a rapid and accurate method of measuring spectral sensitivity in particular regions of stomatopod compound eyes. This is an important goal, since the stomatopods may have the most complex visual systems, in terms of peripheral design, in existence; the design is particularly well-suited for color vision (Marshall, 1988).

If color vision exists in stomatopods, it would depend on two unique features of the compound eyes. The first is the multiple overlap of receptive fields of different ommatidia, resulting both from the triple redundancy of the central band and more peripheral retinal regions (Exner, 1891; Horridge, 1978; Schiff *et al.*, 1985, 1986a, b; Cronin, 1986; Schiff and Candone, 1986), and from the complete sharing of receptive fields by the six ommatidial rows in any column of the central band (Horridge, 1978; Marshall, 1988). Second, ommatidia in two of the individual rows of the central band of gonodactyloid stomatopods contain intrarhabdomal colored filters at two separate levels. Like the oil droplets in retinal cones of reptiles and birds, these filters would shift and narrow the spectral sensitivity functions of the photoreceptors lying under them (Hardie, 1988; Marshall, 1988). Therefore, even with a single visual pigment in all rhabdoms, as is the case with *Squilla empusa* (Cronin, 1985), multiple spectral sensitivity functions are expected and the visual field overlaps would permit hue discrimination to occur. In fact, recent unpublished work suggests that many visual pigments exist in the retinas of gonodactyloid stomatopods.

The argument presented in the previous paragraph strictly applies only to the main rhabdom, formed by the fused rhabdomeres of retinular cells 1–7. Crustaceans generally have a single visual pigment, of peak absorption near 500 nm, in the main rhabdoms of all ommatidia and a second one, absorbing at considerably shorter wavelengths, in the overlying rhabdomere of the 8th retinular cell (Cummins and Goldsmith, 1981; Martin and

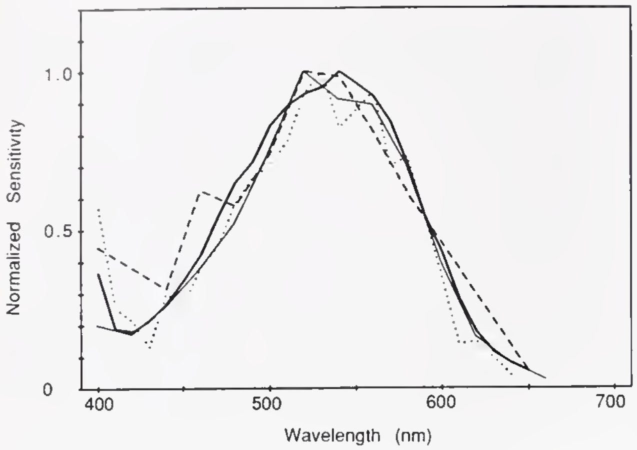

Figure 6. A comparison of the results of the four techniques. Dark solid line: scan technique. Light solid line: criterion technique. Dotted line: interpolation technique. Dashed line: Ramp technique.

Mote, 1982; see also Cronin, 1985; Cronin and Forward, 1988). Most stomatopods also have the 8th cell in all ommatidia (Waterman, 1981; Marshall, 1988), and possess excellent sensitivity to ultraviolet (UV) wavelengths (Schiff, 1963; Cronin, 1989). The presence of UV receptors with sensitivity extending into the spectral range we studied was probably the cause of the large variation of sensitivity we observed at 400 nm.

Stomatopods are brightly colored and display their colors prominently in intraspecific and interspecific communication (Caldwell and Dingle, 1975). In *Gonodactylus oerstedii*, colors are known to play a role in territorial behavior (Hazlett, 1979). Like many stomatopod species, *G. oerstedii* inhabits shallow, well-lit water, and is active during the daytime (Dominguez and Reaka, 1988). There is every reason to expect that the ability to recognize hue exists in some stomatopods, particularly in the gonodactyloids.

In previous intracellular optical investigations of *G. oerstedii*, evidence has been found that only one long-wavelength spectral mechanism exists in the peripheral retina (Cronin, 1989). In contrast, the tiering of rhabdoms in four of the rows of the central band would be expected to produce up to four dichromatic, long-wavelength mechanisms there (Marshall, 1988). For these reasons, we selected the medial peripheral ocular region for this first study.

Results clearly suggest a single receptor class that responds identically to all four experimental approaches (Fig. 6). This function peaks near 540 nm, has a half bandwidth of 125 nm, and has the typical form of a spectral sensitivity function produced by a single visual pigment.

Does the measured function express the true spectral sensitivity of the photoreceptors of peripheral ommatidia? Several lines of evidence suggest that it does. The intracellular optical response is almost certainly coupled to photopigment absorption, at least at long wavelengths, in numerous species of insects (Stavenga, 1979; Bernard and Stavenga, 1979; Bernard and Wehner, 1980; White *et al.*, 1983; Weyrauther, 1986). Crustacean retinular cells have mobile pigment granules that respond to visual stimulation (Ludolph *et al.*, 1973; Olivo and Chrismer, 1980); it is probably these mobile pigments that bring about the measured reflectance changes (Cronin, 1989). Therefore, the responses we measured almost certainly originate in the visual cells and represent the true visual sensitivity.

The close correspondence of results of the various methods (Fig. 6) indicates that any of the methods may be used in the future. Clearly, the spectral scan technique is the most desirable for general use, since it is not only faster by almost an order of magnitude, but also produces the most accurate spectra. Scanning has also proven superior in electrophysiological work (Franceschini, 1984; Menzel *et al.*, 1986).

While scanning is justified for work with a single receptor mechanism, it may have serious drawbacks when applied to multiple receptor classes within a single eye region. This is because the sequence of wavelengths may affect the separate classes differently. For instance, separate chromatic adaptations of the classes may occur while the scan is in progress, but at different parts of the scanned spectrum. Another, somewhat analogous effect may occur with a single receptor class if the scanning intensity is sufficient to cause photopigment conversion; the ratio of photopigment (rhodopsin) to product (metarhodopsin) would then vary throughout the scan. Such effects are generally revealed by a change in shape of the measured function for scans in different directions. In our work, the spectral shapes measured in both directions were identical, providing no evidence either for multiple receptor classes in the scanned regions or for alterations in the rhodopsin/metarhodopsin mixture content.

It is not always knowable in advance whether spectral scanning is justified for use with the photoreceptors of a particular eye region. In such cases, the criterion technique is the best alternate technique of choice. It is as efficient as the other non-scanning techniques, and can be used to test the response at selected wavelengths for relative sensitivity, or for the changes in response waveform or sensitization that are generally associated with multiple receptor classes (Cronin, 1989).

The overall results suggest that spectral sensitivity in this eye region is conferred by a single, unfiltered rhodopsin. The function peaks smoothly, falls steeply at longer wavelengths, and declines more gradually at shorter ones. However, the bandwidth at half-maximum (125

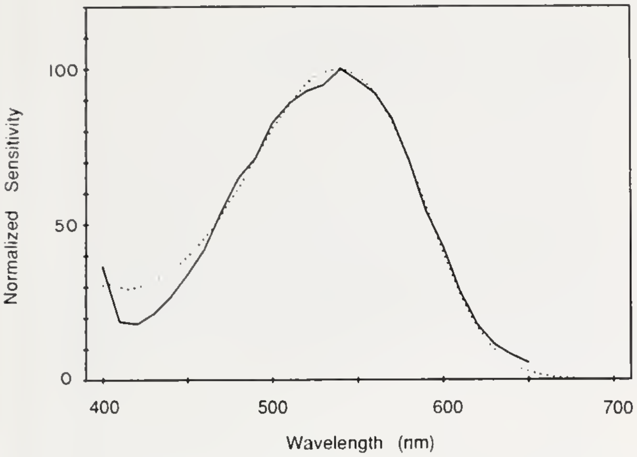

Figure 7. Solid line: average results of spectral scan experiments. Dotted line: computed absorptance spectrum for a rhodopsin having a 537-nm maximum and a peak absorbance of 0.5. The dotted curve is normalized to its maximum, for comparison to the sensitivity function.

nm) is about 25% broader than the 102-nm half bandwidth of the absorption spectrum of a rhodopsin in this spectral location (The absorption spectrum was calculated using an 8th-order polynomial developed by G. D. Bernard and kindly provided by him.) The discrepancy is easily resolved by considering self-screening by the rhodopsin, an effect that broadens the absorption function. In Figure 7 the average curve generated by spectral scanning is plotted together with a computed absorption curve for a rhodopsin peaking at 537 nm and with a maximum density of 0.5. The match between the curves is excellent. In this ocular region, *G. oerstedii* has a sensitivity maximum almost 20 nm to longer wavelengths than that of the squilloid, *Squilla empusa*, which peaks at 520 nm (Trevino and Larimer, 1969).

The techniques described here will now be applied to measure the spectral sensitivities of the various ommatidial rows of the central band. Learning whether the stomatopods possess the diversity of color receptors they seem capable of making will be a demanding, but exciting, task.

Acknowledgments

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Amino Acid Uptake and Metabolism by Larvae of the Marine Worm *Urechis caupo* (Echiura), a New Species in Axenic Culture

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Abstract. Axenic (bacteria-free) larval cultures of the marine echiuran worm, *Urechis caupo*, were reliably obtained by aseptically removing gametes directly from the gamete storage organs. Trochophore larvae only removed neutral amino acids from seawater as measured by high-performance liquid chromatography (HPLC). There was no detectable uptake, as measured by HPLC, of acidic or basic amino acids. Kinetic analysis showed that the transport system for alanine in 4-day-old larvae had a K_t of 4–6 μM and a J_{max} of 9–10 pmol larva^{-1} h^{-1}. Following a 50-min exposure, the majority of the radioactivity (95%) from ^{14}C-alanine was found in the trichloroacetic acid-soluble fraction. Very little label appeared as acid-insoluble material, and there was no detectable lipid biosynthesis from ^{14}C-alanine. Approximately 12% of the total alanine transported was released in the form of $^{14}CO_2$. Thin-layer chromatography of intracellular free amino acid pools demonstrated that aspartic acid and glutamic acid were radiolabeled from the alanine precursor. A comparison of the energy acquired from the transport of alanine, with the metabolic rate of 4-day-old larvae, revealed that 51% of the metabolic demand could be provided by the transport and complete catabolism of this single amino acid at a concentration of 595 nM in seawater.

Introduction

Planktotrophic (feeding) larvae of marine invertebrates must obtain food from the environment in order to supply energy for growth and metabolism (Thorson, 1946; Mileikovsky, 1971). These larvae possess anatomical adaptations to concentrate and clear particles from seawater (Strathmann, 1971; Strathmann *et al.*, 1972). However, adaptations for energy and nutrient acquisition need not exist only for the capture of particulate food. Larvae have a large surface area to volume ratio owing to their small size. Structural elaborations for locomotion and particle capture also increase the surface area (*e.g.*, molluskan velum, echinoderm ciliated bands). The total surface area of the epithelium is further enhanced by the presence of an apical brush border on certain cells (*e.g.*, Waller, 1981; Amieva and Reed, 1987). In addition to the anatomical modifications used for the capture of particles, both larval and adult soft-bodied marine invertebrates can take up dissolved organic material (DOM) directly from seawater across their body-wall (see review by Stephens, 1988).

Uptake of DOM by larvae has been primarily studied as the fluxes of free amino acids from seawater. To date, using a variety of analytical techniques, amino acid transport has been demonstrated for a number of planktotrophic larvae. Larvae of the annelids *Nereis virens* and *Neanthes arenaceodentata* accumulate radioactivity when exposed to ^{14}C-labeled amino acids in seawater (Bass *et al.*, 1969; Reish and Stephens, 1969). Amino acid influx and net flux has been reported for plutei of two species of echinoid echinoderms (*Strongylocentrotus*

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Unusual abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DOM, dissolved organic material; HPLC, high-performance liquid chromatography.

purpuratus and *Dendraster excentricus*) (Manahan *et al.*, 1983; Davis and Stephens, 1984).

Manahan (1983) examined the biochemical fate of ^{14}C from transported amino acids in two bivalve veligers, *Crassostrea gigas* and *Mytilus edulis*. The patterns of carbon assimilation were similar following a 100-min exposure to 0.5 μM ^{14}C-amino acid for *C. gigas* (glycine) and *M. edulis* (alanine). The majority of the radioactivity (75%) in the larva was localized in the cold trichloroacetic acid (TCA)-soluble fraction, 20–25% was associated with the TCA-insoluble pellet, and virtually no label was found in the lipid fraction (<2%). The production of $^{14}CO_2$ by *C. gigas* larvae, measured in parallel, represented 33% of the total glycine transport (the sum of isotope in the larva and respired radioactivity).

Based on these published accounts, most planktotrophic larvae should have the ability to remove amino acids from seawater and incorporate the acquired substrates in metabolism. However, quantitative interpretation of the rate of substrate transport and metabolism has been hampered by the fact that the majority of these experiments were conducted in the presence of bacteria. Heterotrophic bacteria can take up amino acids and other forms of DOM from seawater and use these compounds for growth and metabolism (Williams, 1975). Hence, the metabolic activity of bacteria may confound the results of studies on the ability of larvae to transport and metabolize amino acids. For instance, in adult sea urchins (*Strongylocentrotus droebachiensis*) the activity of intestinal (surface-adherent) bacteria appears to convert radiolabeled glucose to "essential" amino acids (Fong and Mann, 1980). Attempts have been made to eliminate bacteria from larval cultures by the addition of antibiotics (Millar and Scott, 1967), but the effectiveness of antibiotic treatments on marine bacteria is unpredictable and deleterious effects on larvae have been reported by D'Agostino (1972) for the crustacean *Artemia salina*. Recent advances in culturing techniques now allow the production of axenic larval suspensions without the need for antibiotics (Langdon, 1983). Aseptic collection and combination of oocytes, eggs, and sperm has provided axenic larval cultures of *Crassostrea gigas*, *S. purpuratus*, and *Dendraster excentricus* (Langdon, 1983; Manahan *et al.*, 1983; Davis and Stephens, 1984). These three species, representing two phyla, are the only ones for which studies of amino acid transport by larvae from seawater have been conducted under axenic conditions. To evaluate whether patterns of amino acid uptake are the same for all larvae, irrespective of phylogenetic affinity, or if significant differences exist between larvae from different phyla, we have developed a technique that allows the production of axenic suspensions of trochophore larvae of the echiuran worm, *Urechis caupo*.

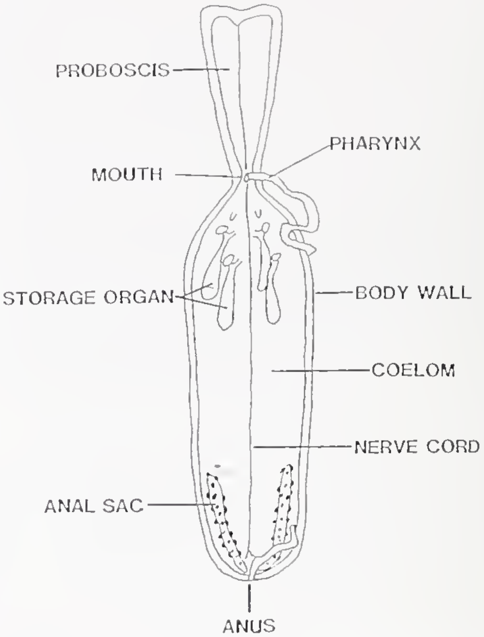

Figure 1. Ventral view of a generalized echiuran worm, drawn to display the location of the gamete storage organs (redrawn from Gould-Somero, 1975).

The echiuran, *Urechis caupo*, MacGinitie and Fisher, 1928, has a high fecundity and easily accessible gametes (Gould-Somero, 1975). The oocytes of this species have been previously used to describe biochemical changes pre- and post-fertilization (*e.g.*, Gould, 1969a, b). *U. caupo* is dioecious and gametogenesis in both sexes occurs within a spacious coelomic cavity. Mature spermatozoa and oocytes are segregated from the general cellular constituents of the coelomic fluid by three pairs of ciliated funnels, and each ciliated funnel is confluent with a gamete storage organ (Fig. 1). These storage organs can be excised allowing easy manipulation of fertilizable gametes.

We report here the development of a technique for aseptic removal of gametes and fertilization that results in axenic cultures for larvae of the echiuran worm, *Urechis caupo*. Axenic larvae were used to measure (i) rates of amino acid uptake by larvae, (ii) biochemical fates of a transported substrate, and (iii) larval metabolic rates.

Materials and Methods

Animal culture

Urechis caupo adults were obtained from Sea Life Supply Co. (Sand City, California) and maintained at 15–17°C in filtered seawater (0.2 μm, pore size, Nuclepore). Adults were removed from the tank and narcotized in 3.1% $MgCl_2$ (w/v) for 30–40 min prior to manipulation.

An incision was made through the body wall in the anterior one-quarter of a worm and continued anteriorly to the level of the proboscis, exposing the coelomic cavity. Each storage organ was clamped with a hemostat and removed by excision. All further manipulations were done, using autoclave-sterilized glassware, in a sterile transfer hood with laminar air flow (Labconco, Inc.). Each storage organ was blotted with a tissue to remove excess fluid carried over with the gamete storage organ. The storage organ was then placed in two sequential 45-s washes in 75% ethanol. Following the second ethanol wash, the storage organ was placed in sterile seawater for 45 s. Hereafter, "seawater" refers to natural seawater which was passed through a 0.2 μm (pore size, Nuclepore) filter, then autoclaved. The gametes were removed and collected by one of two methods depending on the size of the storage organs. Large storage organs were placed on tissue paper, swabbed with 75% ethanol, and the gametes removed with a flame-sterilized syringe needle. Small storage organs were opened with a flame-sterilized scalpel and the gametes collected in a beaker of seawater. Following insemination, the fertilized oocytes were placed in a 6-l Erlenmeyer flask and maintained at a temperature of 15°C. No particulate food (phytoplankton) was provided to the larvae in order to maintain axenic cultures. All experiments were conducted at 15°C.

To collect axenic trochophore larvae for experiments, the contents of a culture flask were aseptically siphoned at a slow rate onto an autoclave-sterilized 44-μm polyester mesh within a transfer hood (laminar air flow). The larvae were placed in a graduated cylinder and the larval concentration was determined by triplicate counting of known aliquots from the larval suspension.

Tests for bacteria

One ml samples of fertilized oocyte suspensions, and seawater from larval cultures, were aseptically placed in 3 ml of a sterile enriched-seawater broth (Ruby *et al.*, 1980). We assayed for contaminating bacteria by monitoring the turbidity of the seawater broth, and by further examination with 4',6-diamidino-2-phenylindole (DAPI) staining and epifluorescent microscopy (Porter and Feig, 1980). DAPI is a DNA-specific fluorescent stain that allows the detection of bacteria in the seawater broth. To test for false negative results, 1 ml of nonsterile seawater was added to the broth and assayed as described above.

Net uptake of amino acids

For experiments designed to measure the uptake of amino acids by larvae, a sufficient volume of the larval suspension was added to seawater in a sterile flask (final volume, 100 or 200 ml) to produce a larval concentration of 150–250 larvae/ml. Thirteen amino acids (made from crystalline powders, Sigma Chemical Co.) were added as a mixture to the larval suspension, producing a known amino acid concentration that ranged from 100 n*M* to 250 n*M* per individual substrate, depending on the experiment. As trochophore larvae of *Urechis caupo* swim vigorously, the experimental flask did not require continuous mixing throughout the experiment. In parallel with the uptake experiments, the amino acid mixture was added to an identical flask containing no larvae. This flask served as a control for changes in substrate concentration attributable to surface adsorption to the flask. At regular time intervals, a 500-μl sample was removed, gently passed through a 0.2-μm (pore size) polycarbonate filter held in a 13-mm filter housing (Nuclepore), and frozen. Depletion of individual amino acids from the medium was determined using high-performance liquid chromatography (HPLC). Amino acids in seawater were derivatized with *o*-phthaldialdehyde (Lindroth and Mopper, 1979) and the fluorescent derivatives separated using a sodium acetate-based buffer system (Jones *et al.*, 1981) on an Ultrasphere ODS column (4.6 cm × 7.5 mm; 3 μm particle size). The eluent profile and HPLC equipment are described elsewhere (Manahan, 1989).

The concentrations of amino acids used in our experiments were below the half-saturation constant (the K_t) of 4–6 μM for a representative neutral amino acid (see Table I). Thus, the depletion rates of individual amino acids are first-order with respect to substrate concentrations and, therefore, are nonlinear as a function of time (see Fig. 4). Any calculation of uptake rates based on end-point analyses would, under these circumstances, be invalid. At each sampling interval, the concentration for each of the 13 individual amino acids was ln-transformed to yield a linear plot. The rate of amino acid uptake by larvae was calculated from the rate of substrate depletion using the first-order depletion constant, "k", where $k = (\ln [S]_o - \ln [S]_t)/t$ (see Segel, 1976, p. 227). The depletion constant was calculated from the slope of a least-squares linear regression analysis of ln-transformed substrate concentrations with time. Values for the initial substrate concentration ($[S]_o$), and the final substrate concentration ($[S]_t$), were calculated from the regression equation based on all data points. The rate of net flux, expressed as pmol amino acid larva^{-1} h^{-1}, was calculated by multiplying the amount of each amino acid present in the mixture (*e.g.*, 20 nmol/200 ml, $[S] = 100$ n*M*) by its respective depletion constant (k), and then dividing each rate by the total number of larvae in the flask.

To determine whether the disappearance of ^{14}C-labeled alanine from the medium actually represented the net substrate flux into a larva, the change in the amount of total alanine (^{12}C and ^{14}C), as a function of time, was determined by direct chemical measurement with HPLC. The rate of influx of ^{14}C-alanine was measured by monitoring the change in the amount of ^{14}C in the medium with isotope techniques. Both the HPLC and the ^{14}C measurements were made on different aliquots of the same samples. At the start of each experiment, a known amount of ^{14}C-alanine (168 μCi/μmol, New England Nuclear) was added to the same flask used to determine, by HPLC measurement, the rates of amino acid uptake into larvae. Samples of seawater were removed and filtered as described above. The depletion of total alanine in the medium (measured by HPLC) was compared to the disappearance of ^{14}C-labeled alanine (measured by liquid scintillation counting). The samples used for detection of radioactivity were acidified ($pH < 2$) for 1 day to volatilize any $^{14}CO_2$, prior to the addition of 5 ml of scintillation cocktail (Scinti-Verse, Fisher Scientific).

Kinetics of alanine transport

A known number of 4-day-old larvae was transferred into each of ten 20-ml vials (10 ml final volume). Each vial was used to measure the rate of alanine transport at a specific substrate concentration ranging from approximately 0.5 to 100 μM. To determine both the K_t and the J_{max} for the alanine transport system, the substrate concentrations that were used had to be greater than the reported concentrations of alanine in seawater (*e.g.*, Mopper and Lindroth, 1982). To accurately measure low amounts of radioactivity, relatively high concentrations of larvae (*ca.* 240/ml) were used in each experiment. To compensate for these concentrations of larvae, short time course experiments were performed. A 7–8 min experiment was conducted for each substrate concentration. At sampling intervals of approximately 1 min, a 500-μl sample was removed and layered onto 500 μl of silicone oil (Versilube F-50, General Electric Co.) in a 1.5-ml microfuge test tube. The larvae were separated from the medium by centrifugation (Beckman Model "E" microfuge, 12,500 × *g*). Following centrifugation, the supernatant and oil were removed, and the pellet of larvae collected by cutting off the bottom of the centrifuge tube. The larvae were immediately placed in a 6-ml scintillation vial and 500 μl of tissue solubilizer (Scinti-Gest, Fisher Scientific) was added. The tissue was digested for 48 h before the radioactivity was determined. Sample radioactivity was corrected, if necessary, for quenching by the addition of a ^{14}C-toluene internal standard. The slope of the regression line for each time course experiment was used to calculate the rate of alanine transport (pmol larva^{-1} h^{-1}).

Comparison of transport rates for alanine, arginine, and glutamic acid

To compare the relative rates of transport, sibling larvae, from a 2-day-old xenic culture, were exposed to either an acidic, basic, or a neutral amino acid, each at a substrate concentration of 1 μM. Radiochemicals were purchased from either New England Nuclear (^{14}C-alanine, 168 μCi/μmol; or I.C.N. (^{14}C-arginine, 270 μCi/μmol; ^{14}C-glutamic acid, 245 μCi/μmol). The procedures used to determine the transport rate for each substrate were identical to those described above for the kinetics of alanine transport.

Metabolic fate of alanine

In another series of experiments, the biochemical fate of the ^{14}C-label in the larvae was determined following the transport of ^{14}C-U-alanine (1 μCi/10 ml) from a substrate concentration of 595 nM. During the course of 1 h, two 500-μl samples containing larvae were removed from the 10-ml vial at approximately 10 min intervals. To measure the influx of radiolabeled alanine, one sample was processed and the larvae collected as described above (see "kinetics of transport"). The other sample of larvae was gently collected on a polycarbonate filter (25 mm diameter, 5.0 μm pore size, Nuclepore), and washed twice with isothermal seawater to separate the larvae from excess radioactivity in the medium. Similar filtering procedures did not cause any efflux of ^{14}C-alanine, or any rupturing and loss of ^{12}C-alanine, from larvae (see Fig. 4). Immediately after the washings, the larvae were killed by freezing on dry ice. The filters were lyophilized for at least 3 h to remove any residual water and the larval tissue was disrupted by sonication in 1 ml of distilled water (Sonics and Materials Brand, Model VC 40 fitted with a microprobe). A 50-μl aliquot of the homogenate was taken, dissolved in tissue solubilizer, and measured to determine the total radioactivity in the homogenate. A known volume of the homogenate was separated into (i) the insoluble material in cold trichloroacetic acid (TCA), (ii) TCA-soluble, and (iii) chloroform-soluble fractions, following Mann and Gallagher's (1985) modification of the procedures described in Holland and Gabbott (1971). The following steps were added to eliminate isotope carry-over during fractionation: (a) the TCA-pellet was washed three times with 100% diethyl ether to remove residual TCA and heated to dryness, (b) the chloroform extract was heated to dryness at 100°C, and (c) both TCA-insoluble and the chloroform-extractible fractions were dissolved in tissue solubilizer for 24 h prior to the addition of scintillation cocktail. The rate of production of $^{14}CO_2$ from axenic larvae was measured using the techniques of Manahan (1983). The fate of ^{14}C-alanine in the free amino acid pools of larvae was observed using two-dimensional thin-layer chromatography (Jones and Heathcote, 1966) of ethanol extracts (75%, v/v). An aliquot of the extract (2 μl) was spotted onto a 10 cm × 10 cm cellulose plate (0.1 mm thick, EM Science Brand) and the radioactivity visualized after exposure to X-ray film (XOMAT AR, Eastman-Kodak Co.).

Metabolic rates of larvae

Oxygen consumption was measured using a Clark-type polarographic oxygen electrode system, consisting of a Strathkelvin Instruments oxygen meter (Model 781), a micro-electrode (#1302), and a microrespiration chamber (RC200). The respiration chamber was calibrated to 100 μl total volume and maintained at 15°C ± 0.02°C by a water bath (Model RDL 20, Precision Instruments). Prior to experiments, the stability and self-consumption rate of the electrode were determined in isothermal seawater. After a 2-min equilibration period, following the addition of larvae, the change in oxygen tension (mm Hg) within the respiration chamber was recorded every min for at least 21 min. At the completion of each experiment ($n \geq 3$), the larvae were removed and counted (*ca.* 100 to 200 per experiment). The electrode was calibrated to zero oxygen tension by deoxygenating seawater with excess sodium thiosulfite or sodium sulfite. Calibration of the meter reading (mm Hg) with molar oxygen concentration was made by measuring the oxygen tension in a 300-ml BOD bottle filled with isothermal seawater, and then chemically determining the molar oxygen concentration using the Winkler titration method (Parsons *et al.*, 1984).

Results

Culturing the larvae of Urechis caupo *under axenic conditions*

Aseptic removal of mature gametes from *Urechis caupo*, and subsequent fertilization, yielded axenic larvae. Approximately 80% of the cultures produced in this manner tested negative for the presence of bacteria, even after 3 years of exposure to the enriched seawater broth. For axenic cultures, DAPI-stained gametes, larvae, and culture water all tested negative for bacteria. All broths inoculated either with (i) nonsterile seawater or (ii) xenic suspensions of gametes or larvae, were visibly cloudy after 24–48 h, eliminating the possibility of false-negative results for the axenic cultures. Examination of sibling axenic and xenic larvae by light microscopy revealed no distinguishable difference in larval morphology.

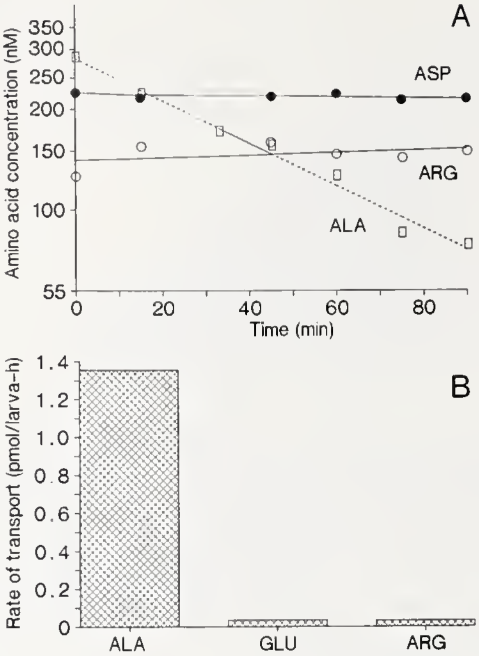

Figure 2. (A) Net flux of representative acidic, basic and neutral amino acids by 2-day-old *Urechis caupo* trochophore larvae (265 ml^{-1}), as measured by high-performance liquid chromatography (HPLC). The slopes of the regression lines for the net flux rates of aspartic acid (solid circles) and arginine (open circles) were not statistically significant from zero; the uptake of alanine (rectangles) was statistically significant ($P \leq 0.001$). (B) The rate of transport by sibling larvae of ^{14}C-alanine ($r^2 = 0.99$), ^{14}C-glutamic acid ($r^2 = 0.89$), and ^{14}C-arginine ($r^2 = 0.89$), each measured separately at a substrate concentration of 1 μM (258 larvae/ml).

Net uptake from amino acid mixtures

Trochophore larvae of *Urechis caupo* took up only neutral (polar and nonpolar) amino acids from seawater. There was no statistically significant influx of either acidic or basic amino acids from the experimental mixture of 13 individual amino acids (Fig. 2A). This result was observed for three other cultures of axenic larvae, obtained from independent spawnings. The possibility that competition between the substrates caused this observation was eliminated by comparing the rates of transport for alanine, arginine, and glutamic acid in the absence of other amino acids (Fig. 2B). From a substrate concentration of 1 μM, where the transport rate of each of the three substrates was measured separately, alanine was transported at a rate of 1.35 pmol larva^{-1} h^{-1}; glutamic acid at a rate of 0.03 pmol larva^{-1} h^{-1}; and arginine was transported also at a rate of 0.03 pmol larva^{-1} h^{-1}. Thus, the rates of transport of Arg and Glu were only 2.1% of that for alanine at an identical concentration. Figure 3 shows the rates of uptake for each of 13 individual amino acids. The total rate of uptake for the 9 neutral

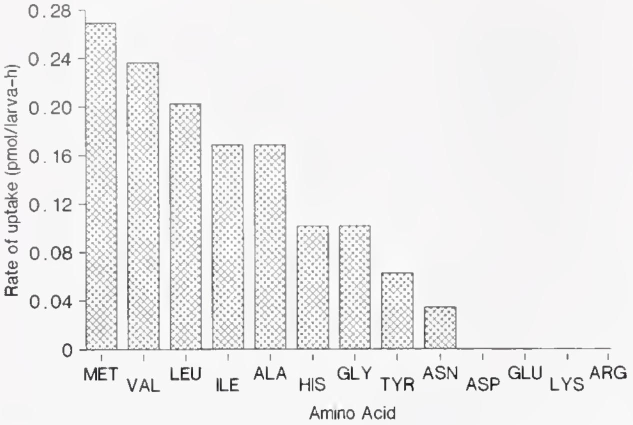

Figure 3. Depletion of 13 amino acids from the medium in the presence of trochophore larvae of *Urechis caupo* (250 larvae ml^{-1}) determined by high-performance liquid chromatography. The rate of amino acid uptake was calculated from the first order depletion constant (k) (see text). The concentration of each amino acid at the start of the experiment was 100 nM; where no histogram bars are presented the change in the ln-transformed substrate concentrations were not statistically significant from a slope of zero. The r^2 values for all statistically significant rates were >0.90.

amino acids (His is neutral at the pH of seawater) was 1.34 pmol amino acid larva^{-1} h^{-1}. In the absence of larvae, there was no detectable change in the concentration of any amino acid in the flask.

The influx of alanine equaled the net substrate flux as shown in Figure 4. The rate of influx, measured as the depletion of ^{14}C-alanine from the medium, was 0.23 pmol larva^{-1} h^{-1} ($r^2 = 0.99$); the net flux of alanine, measured by HPLC, was 0.24 pmol larva^{-1} h^{-1} ($r^2 = 0.99$). The starting substrate concentration was 207 nM. A comparison of the regressions for both these rates showed that there was no statistically significant difference between these rates (VR = 0.14, $F_{.05[1,10]} = 4.96$).

Kinetics of alanine transport

A graph showing the influence of substrate concentration on the rate of transport produced a rectangular hyperbola (Fig. 5). Linear transformations (Eadie-Hofstee) were characteristic of Michaelis-Menten kinetics (Fig. 5, see inset). The kinetics of alanine transport by 4-day-old trochophores were examined for both axenic and xenic larvae (nonsibling cultures). Axenic larvae had a K_t of 4 μM for alanine and a J_{max} of 9–10 pmol larva^{-1} h^{-1} (Table I). Nonaxenic larvae had very similar kinetics, with a K_t of 5–6 μM and a J_{max} of 9–10 pmol larva^{-1} h^{-1} (Table I).

Metabolic fate of alanine in larvae

From a concentration of 595 nM, the influx of ^{14}C-U-alanine into 4-day-old larvae was 1.72 pmol larva^{-1} h^{-1}

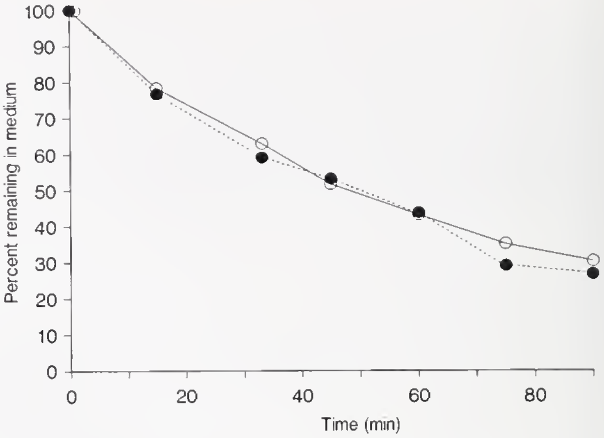

Figure 4. A comparison of influx and net flux of alanine from the medium in the presence of 1-day-old axenic *Urechis caupo* larvae (297 larvae ml^{-1}). Net flux was determined by HPLC (solid circles), and influx was measured by isotope techniques (open circles). The concentration of alanine at the beginning of the experiment was 207 nM.

(Table II). A second culture of 7-day-old larvae transported ^{14}C-U-alanine (595 nM) at a rate 1.97 pmol larva^{-1} h^{-1} (Table II). For both cultures, the majority (>95%) of the radioactivity was recovered in the TCA-soluble pool and there was no detectable radioactivity in the lipid fraction. Simultaneous measurement of $^{14}CO_2$ production, expressed as alanine equivalents, showed that 0.25 pmol and 1.10 pmol larva^{-1} h^{-1} were released by 4- and 7-day-old larvae, respectively.

Autoradiographic analysis of TLC-separated amino

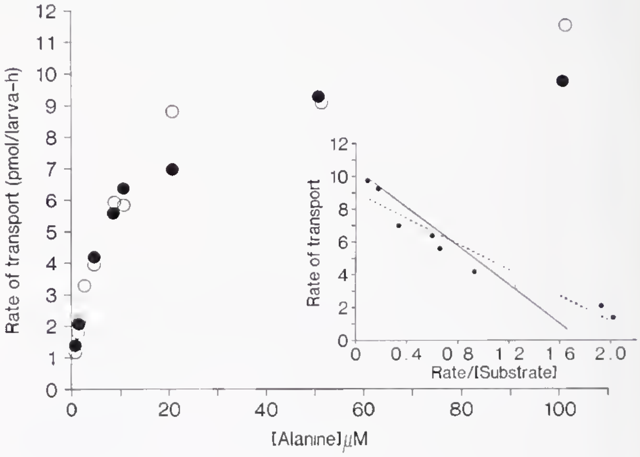

Figure 5. Effect of substrate concentration on the rate of alanine transport by axenic (solid circles) and xenic (open circles) 4-day-old *Urechis caupo* larvae. The concentration of larvae was 119 ml^{-1} (axenic experiments, n = 8) and 127 larvae ml^{-1} (xenic experiments, n = 10). Each data point (= n) represents the slope of a single time course experiment (all r^2 values > 0.95). The inset shows Eadie-Hofstee plots of the data.

Table I

A comparison of kinetic constants for alanine transport by axenic and xenic Urechis caupo *larvae (4-day-old)*

Larval condition Axenic larvae	Linear transformation Eadie-Hofstee	 Lineweaver-Burke
K_t (μM)	3.9	4.3
J_{max} (pmol larva^{-1} h^{-1})	9.0	10.0
r^2 (n = 8)	0.92	0.97
Nonaxenic larvae		
K_t (μM)	5.9	5.0
J_{max} (pmol larva^{-1} h^{-1})	10.5	9.1
r^2 (n = 10)	0.93	0.99

ANOVA	SS	df	MS	VR	F_{05}
Between slopes (K_t)	6.50	1	6.5	6.50**	4.60
Between constants (J_{max})	0.27	1	0.27	0.27ns	
Residual	14.40	14			
Total	178.17	17			

**Significant at the 99% level.

acid pools of larvae showed that most of the alanine remained intact after a 1-h exposure. However, carbon from ^{14}C-alanine also appeared in both glutamic acid and aspartic acid.

The metabolic rate of Urechis caupo *larvae*

The metabolic rate of axenic 4-day-old larvae was 10.1 ± 0.41 pmol O_2 larva^{-1} h^{-1} (mean ± 1 SE; n = 3). These rates of oxygen consumption were determined for larvae which were from the same culture as those that were used to determine rates of ^{14}C-alanine influx and metabolism (see Table II).

Discussion

A reliable method has been developed to produce axenic larvae of the marine echiuran worm *Urechis caupo.* Cultures of axenic larvae have been used to determine (i) the uptake rates of amino acids from seawater, (ii) the fate of a specific substrate following transport, and (iii) the metabolic rate. This has allowed a direct comparison, in the absence of competing species, of the contribution from a specific fraction of the DOM pool to the metabolism of *U. caupo* larvae.

Axenic *Urechis caupo* larvae only took up neutral amino acids from seawater, as measured by HPLC (Figs.

Table II

Metabolic fate of the ^{14}*C-label following transport of* ^{14}*C-U-alanine by axenic* Urechis caupo *larvae*

	Uptake	Metabolic components CO_2	 % TCA-insoluble[a]	 % TCA-soluble[b]	 % Lipid[c]
4-day-old:					
Rate					
(pmol Ala larva^{-1} h^{-1})	1.72	0.25	5	95	0
r^2 (n)[d]	0.99 (6)	0.96 (5)			
7-day-old:					
Rate					
(pmol ala larva^{-1} h^{-1})	1.97	1.10	3	97	0
r^2 (n)[d]	0.99 (5)	0.96 (6)			

[a] Percent of total radioactivity appearing in the cold TCA-insoluble fraction (macromolecules).

[b] Percent of the total radioactivity appearing in the cold TCA-soluble fraction (small molecular weight compounds).

[c] Percent of the total radioactivity in the larva contained in the chloroform-extractible fraction (lipid).

[d] "n" equals lthe number of samples used to determine the presented rate.

2A, 3). Although significant removal from the medium of either acidic or basic amino acids could not be measured, larvae do have a small transport capacity for glutamic acid and arginine, at only 2% that of the alanine transport rate (Fig. 2B). This difference in the transport capacity for acidic, basic, and neutral amino acids has also been reported for pluteus larvae of the sea urchin *Strongylocentrotus purpuratus* (Manahan *et al.*, 1983) and the sand dollar *Dendraster excentricus* (Davis and Stephens, 1984). The rate of disappearance of ^{14}C-alanine (influx) from the medium, in the presence of axenic larvae, is an accurate measurement of net flux (Fig. 4). The appearance of alanine in the tissue, therefore, represents a net gain to the larva (*cf.* Johannes *et al.*, 1969).

Kinetic analysis of the rate of alanine transport into *Urechis caupo* trochophores indicated that the transport system is adapted to function with maximal efficiency at relatively low substrate concentrations (K_t = 4–6 μM, Table I). The y-intercepts (J_{max}) for the kinetic data, calculated from the regression of the transformed data (Eadie-Hofstee), were not statistically different between 4-day-old axenic and xenic larvae (see ANOVA, Table I). However, the values for K_t were statistically different (VR = 6.50, $F_{.05[1,14]}$ = 4.60). The fact that there was no difference in the maximal rate of alanine transport, and the similarity of the K_t values, suggest that bacteria did not have a significant influence on alanine transport by xenic larvae (Fig. 5, Table I). The kinetic constants for alanine transport by *Urechis caupo* larvae compare favorably with literature values for amino acid transport by other embryos and larvae. Epel (1972) described the kinetics of alanine, glycine, and valine transport in fertilized eggs of the sea urchin *Strongylocentrotus purpuratus*. The kinetics of all three substrates are characterized by both a low K_t (1–2 μM) and a low J_{max} (1.4–2.5 pmol substrate larva^{-1} h^{-1}). Manahan (1983) reported that the veliger larvae of *Crassostrea gigas* had a K_t of 3.7 μM and a J_{max} (25.7 pmol larva^{-1} h^{-1}) for glycine transport. He also measured a similarly low K_t (3.5 μM) and a lower J_{max} (10.1 pmol larva^{-1} h^{-1}) for alanine transport by *Mytilus edulis* veliger larvae. Davis *et al.* (1985) examined the kinetics of serine and leucine transport by 1-day-old *S. purpuratus* embryos and found low values for K_t of 1.3 μM (leucine) and 3.6 μM (serine). The maximal rate of substrate transport was 2.5 and 6.9 pmol substrate larva^{-1} h^{-1}, respectively, for the two amino acids.

Following exposure to ^{14}C-alanine, *Urechis caupo* larvae use this substrate in anabolic and catabolic pathways. The majority (95%) of the intracellular radioactivity was associated with the TCA-soluble pool (Table II). This analytical fraction contains small molecular weight compounds and, thus, may represent an intracellular reservoir of compounds that will be subsequently used in metabolic reactions. In the current experiments, it was not surprising that the amount of radioactivity in the acid-insoluble fraction was low (<5%). Under the experimental conditions used in the present study, the larvae were not fed, and thus a high rate of net protein synthesis was unlikely. However, in veliger larvae of the bivalve *Crassostrea gigas*, which had been fed phytoplankton, Manahan (1983) reported that 20–25% of the radioactivity was localized in acid-insoluble material. The ethanol-extractable amino acids in *U. caupo* trochophores were separated by TLC and those containing the ^{14}C-label were detected with autoradiography. Following a 1-h exposure, the ^{14}C-label from ^{14}C-alanine appeared in alanine, aspartic acid, and glutamic acid. Four-day-old trochophore larvae released 12% of the total transported ^{14}C-amino acid carbon as $^{14}CO_2$. Although the uniform labeling of the isotope precludes any estimate of the precise energy derived from alanine breakdown, it is clear that this substrate is used in catabolic reactions.

If the amount of energy gained from the transport of alanine is compared to the total energy requirements of a larva (oxygen consumption), an indirect measure of the energetic significance of the transported substrates can be made. This comparison makes no assumptions about metabolic coupling between these two processes, but merely provides a means to evaluate the potential input from this mode of nutrient acquisition (Stephens, 1963). At an alanine concentration of 595 nM, trochophore larvae (4-day-old) had a total uptake rate of 1.72 pmol alanine larva^{-1} h^{-1} (Table II). Larvae of the same age, from the same culture, had a metabolic rate of 10.1 pmol O_2 larva^{-1} h^{-1}. The complete combustion of 1.72 pmol of alanine would require 5.16 pmol O_2 (1 mol alanine requires 3 mol O_2). Thus, a transport rate of 1.72 pmol alanine could account for a metabolic rate of 5.16 pmol O_2 larva^{-1} h^{-1}, or 51% of the measured metabolic rate of these larvae (10.1 pmol O_2 larva^{-1} h^{-1}). The measured rate of alanine transport at 595 nM was 1.72 pmol larva^{-1} h^{-1}, which was higher than that calculated from kinetic constants (given in Table I), for axenic larvae at the same substrate concentration (rate = 1.19 pmol larva^{-1} h^{-1}). Nonetheless, for this comparison we feel justified in using the measured value of 1.72 pmol larva^{-1} h^{-1} because both the metabolic rate and the transport rate were determined, on the same day, for the same culture of axenic larvae.

Dissolved organic carbon (DOC) in seawater ranges from 500 to 5800 μg C l^{-1}, and represents nearly ten times the amount of carbon in particulate form (Williams, 1975; Sugimura and Suzuki, 1988). Of the total DOC, free amino acids represent less than 1%, and ambient concentrations in surface seawaters range from less than 10 nM to 500 nM (Christensen and Blackburn, 1980; Mopper and Lindroth, 1982; Carlucci *et al.*, 1984; Fuhrman and Bell, 1985). Near, and within, marine sediments, the values for amino acid concentrations are 1–2 orders of magnitude higher (Clark *et al.*, 1972; Henrichs and Farrington, 1979). Although the concentrations of total free amino acids in seawater may be relatively low, it is clear that *Urechis caupo* larvae have the ability to transport these molecules at rates sufficient to provide a caloric equivalent to 51% of the metabolic rate. Of course, the rate of amino acid transport that is realized in nature will depend on the ambient substrate concentrations in the immediate environment of the larvae. The adults of *U. caupo* are infaunal inhabitants of estuaries and embayments, and their larvae will be exposed to the higher substrate concentrations of DOM found in these habitats. The demonstrated ability of *U. caupo* larvae to acquire amino acids from seawater, to metabolize them, and potentially gain a significant amount of energy, suggests that the capacity to exploit this resource is important. During periods of low particulate food availability, the ability of a larva to use another energy and nutrient source may allow a normal developmental rate to be maintained. Alternatively, if particulate food is not limiting, the additional energy from DOM could be used to accumulate energy reserves.

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Epidermal Uptake of Nutrients in an Unusual Turbellarian Parasitic in the Starfish *Coscinasterias calamaria* in Tasmanian Waters

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Abstract. The parasitic turbellarian *Acholades asteris* (Neorhabdocoela: Acholadidae) lives encysted on the ambulacral tube-feet of the starfish *Coscinasterias calamaria* in Tasmanian waters. Cysts, each containing one worm, generally occur singly as spindle-shaped protrusions from near the bases of the tube-feet; the cyst wall consists of tube-foot epidermis, with nervous tissue, and part of the outer layer of the tube-foot's two-layered connective tissue sheath. The inner layer, musculature, and coelomic epithelium are not involved, and the tube-foot remains functional. The turbellarian lies within the split outer layer with its anterior towards the base of the tube-foot. It lacks mouth, pharynx, and intestine and feeds via its ciliated epidermis on the surrounding collagenous and cellular materials. These are partially digested by enzymes from epidermal and subepidermal glands, products are pinocytosed, and digestion is completed in epidermal phagosomes formed by fusion of pinocytotic vesicles. Food uptake occurs over the entire body surface but especially anteriorly where the epidermis is thicker, deeply invaginated, and underlain by concentrations of gland cells. In approximately 25% of flatworms examined, the epidermis and adjacent parenchyma contained trophozoites and other stages of *Monocystella* sp., an aseptate gregarine whose other, known, species occur in turbellarian alimentary systems.

Introduction

The Turbellaria are typically free-living flatworms but many foreshadow the exclusively parasitic life styles of the Monogenea, Digenea, and Cestoda by living in various types of permanent symbiotic associations with other animals (Jennings, 1971; Cannon, 1986). The nutritional adaptations of many such symbiotic species are now well known and have been reviewed by Jennings (1977, 1988) and Shinn (1981). Feeding patterns include epi- and entozoic predation, ecto- and entocommensalism, entoparasitic utilization of host tissues or metabolites, and various combinations of these. Patterns of digestive physiology are correlated with feeding habits and range from examples identical with those of free-living predators to instances of partial or total dependence on digestive enzymes obtained from the host. However, even in these latter cases the alimentary system usually remains anatomically well developed, apart from the loss of intrinsic gland cells, and in the majority of symbiotic turbellarians it is the principal, if not the only, route along which nutrients enter the body. Epidermal uptake as a supplementary mode of nutrition has not been demonstrated in such species, unlike the comparable situation in digenetic trematodes (Smyth and Halton, 1983). There are no obvious adaptations for absorption or membrane ("contact") digestion; ultrastructural studies of the epidermis in, for example, the umagillid *Syndesmis franciscana* by Holt and Mettrick (1975) and the graffillids *Paravortex cardii* and *P. karlingi* by MacKinnon *et al.* (1981), have not revealed significant differences in the numbers or sizes of the microvilli occurring between the epidermal cilia, or in the occurrence of epidermal folds or invaginations, when these species are compared with a representative range of free-living turbellarians (reviewed by Tyler, 1984). The possibility of supplementary epidermal uptake cannot be dismissed, as Jondelius (1986) reports the occurrence of coated vesicles and similar structures, suggestive of pinocytosis, in

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the distal regions of epidermal cells in the umagillid *Anoplodium stichopi*.

A minority of entoparasitic turbellarians, notably the Fecampiidae and Acholadidae, have lost all traces of an alimentary system from their adult stages. In these instances epidermal uptake of the nutrients necessary for growth and reproduction would seem to be the only possible mechanism. In the Fecampiidae, species of *Fecampia* and *Kronborgia* live in the hemocoel of various crustaceans (Caullery and Mesnil, 1903; Christensen, 1981; Bellon-Humbert, 1983; Shinn and Christensen, 1985), a habitat in which they are bathed in soluble potential nutrients. In *Kronborgia amphipodicola*, the only species for which relevant data are available, the epidermal microvilli are close-set, clavate, and very much longer (up to 3 μm in height) than those of other turbellarians. These features were interpreted by Bresciani and Køie (1970) as adaptations increasing epidermal surface area for either passive or active absorption. Bresciani and Køie suggest that pinocytosis may also occur but found very few apparently pinocytotic vesicles in the epidermal cells.

The fecampiid *Glanduloderma myzostomatis* lives in the mesenchyme of myzostomid annelids (Jägersten, 1942), while the only known member of the Acholadidae, *Acholades asteris*, occurs in the connective tissue of the tube-feet of a starfish (Hickman and Olsen, 1955). In these habitats they obviously have smaller quantities of soluble nutrients available for epidermal uptake than do *Fecampia* or *Kronborgia* spp., and the absence of orthodox alimentary systems precludes direct ingestion of host tissues in the manner seen in tissue-feeding flatworms such as graffillid and pterastericolid rhabdocoels (Jennings and Phillips, 1978; Jennings and Cannon, 1985; Kent and Olson, 1986) and some digenetic trematodes (Smyth and Halton, 1983).

It is clear from this brief survey that much remains to be learned about the nutrition of those entosymbiotic turbellarians that lack recognizable alimentary systems. Therefore, in the present study the tissue-dwelling rhabdocoel *Acholades asteris* has been examined by histological and histochemical methods, and ultrastructurally so far as was possible with the available material, to ascertain the nature of its food and methods of digestion and assimilation.

Materials and Methods

Specimens of the starfish *Coscinasterias calamaria* (Gray) (Asteroidea: Asteriidae) were collected in November 1986 by SCUBA divers from depths of 8–12 m in D'Entrecasteaux Channel off Tinderbox, S.E. Tasmania. Ambulacral tube-feet bearing yellow to orange cysts containing the turbellarian *Acholades asteris* Hickman and Olsen (Neorhabdocoela: Acholadidae) were removed within 6 h of collection and fixed *in toto* in Bouin's fluid, 90% ethanol or 10% formalin buffered to pH 7.0 with 0.1 *M* sodium phosphate and used at 4°C.

The structure and inter-relationships of the tube-foot, cyst wall, and the turbellarian's body wall and associated glands were studied in paraffin wax serial sections, 4 or 8 μm thick, prepared by standard procedures after Bouin or ethanol fixation. The sections were stained by an alcian blue, periodic acid-Schiff (PAS) and orange G trichrome method for glycoproteins and mucosubstances (Pearse, 1972), Curtis's Ponceau S method for collagen, Ehrlich's haematoxylin and eosin, Feulgen's method for DNA, Giemsa's stain, Gomori's aldehyde-fuchsin method for elastin, Heidenhain's iron haematoxylin and metanil yellow, Mallory's trichrome stain, or the PAS method, with amylase controls, for glycogen.

Endopeptidases produced by the turbellarian were detected after fixation in cold neutral formalin, using the indoxyl acetate method for non-specific esterases (Holt, 1958). Tube-feet and attached cysts were incubated in the standard medium, and in media containing specific activators and inhibitors as described by Hassall and Jennings (1975) and Jennings (1985), for 6 h at 20°C and at pH 4.5, 7.0, or 8.5. Specimens were then washed in 0.1 *M* phosphate buffer, dehydrated in graded ethanols, and serially sectioned in paraffin wax at 4 μm.

All sectioning and subsequent procedures were carried out in England after collection of specimens and preliminary processing in Tasmania. Due to unavoidable circumstances, no appropriately fixed material was available for ultrastructural study, other than tube-feet and cysts fixed and held in 10% neutral formalin. Trimmed portions of these were washed in 0.1 *M* phosphate buffer at pH 7.0, post-fixed for 3 h in 3% glutaraldehyde in buffer, washed in buffer, and treated for 1 h with buffered 2% osmium tetroxide. They were then embedded in araldite and sectioned; thin sections were mounted on formvar-carbon films carried on copper slot grids, stained with uranyl acetate and lead citrate, and examined in a JEOL 1200 EX transmission electron microscope. Other sections, 1–2 μm thick, were stained with Azur II and examined using the light microscope.

Results

Cysts containing *Acholades asteris* were found on 11 of the 14 *Coscinasterias calamaria* examined. In these almost all ambulacra bore infected tube-feet and further incidence data were not recorded. The bright yellow or orange cysts usually occur singly as spindle- or pear-shaped protrusions, 4–5 mm in length when mature, attached near the bases of the tube-feet (Fig. 1A). Cysts were not found beyond the mid-regions of the podial columns, nor on the sucker-discs. Occasionally, two or even three cysts may occur on the same tube-foot; this is most likely in the proximal rather than distal portions of the ambulacrum. Infected tube-feet remain fully functional and show the same amounts of extension, retraction, bending, and adhesion as uninfected ones. The cysts show only limited movements, mainly as changes in length and occasional lateral bending; these are caused by movements of the contained flatworm whose shape within the cyst can be seen when viewed against bright transmitted light.

Histological structure of the tube-foot wall and cyst wall

The histological structure of the ambulacral tube-feet in *C. calamaria* (Figs. 1A, B) does not differ significantly from that described for other asteroids. Briefly, the tube-foot wall consists of a thin cuticle overlaying tall columnar epidermal cells interspersed with at least four types of acidophilic and basophilic gland cells. The epidermis rests on a well-organized nervous layer composed of nerve cells and circular and longitudinal nerve fibers. This nervous layer is condensed and thickened along the oral side of the podial column to form a prominent longitudinal strand, the podial nerve. There is a similarly thickened circular strand around the terminal sucker-disc.

Two layers of connective tissue lie below the nervous layer. The outer one is diffuse and consists of longitudinally orientated bundles of collagenous fibers interspersed with cellular elements including rounded or amoeboid cells, with small compact nuclei. The staining properties and appearance of these cells identify them as wandering coelomocytes of the type common in the coelomic cavity of the tube-foot. The collagenous elements stain deeply with the classic Ponceau S method for collagen and to varying degrees with PAS, acid fuchsin, and eosin. Occasional elastin fibers also occur, staining with aldehyde-fuchsin.

The inner layer of connective tissue is narrower than the outer one, is extremely compact, and consists entirely of circularly orientated collagenous fibers. It encircles the muscular layer, which forms the longitudinal retractor muscle, and this in turn is limited by a thin cellular epithelium lining the tube-foot lumen.

The cyst wall (Fig. 1B) is formed from starfish tissue without any contribution from the turbellarian and is essentially an extension of the tube-foot cuticle, epidermis, nervous tissue, and the outermost fibers of the longitudinal connective tissue. The turbellarian is, in effect, inserted within the outer connective tissue layer. The components of the cyst wall are continuous with their counterparts in the tube-foot. Sections of small cysts containing sexually immature turbellarians show that the cyst begins as a small blister-like swelling that increases in size as the tube-foot's epidermal, nervous, and outer connective tissues undergo localized growth to accommodate the growing parasite. As the latter increases in length, differential growth of the cyst wall produces the typical spindle- or pear-shaped cyst.

The orientation of Acholades asteris *relative to the tube-foot*

Transverse sections of infected tube-feet show that cysts containing *A. asteris* never form either under or immediately adjacent to the thickened longitudinal strand of nervous tissue. When only a single cyst is present it is almost always diametrically opposite the strand. When two or three cysts occur the larger and older one, as judged from the condition of the turbellarian's gonads (*A. asteris* is protandrous), is usually opposite the strand with the smaller cyst or cysts nearby but quite separate.

Within the cyst, the turbellarian is always orientated with what is interpreted as its morphologically anterior end directed towards the bases of the cyst and tube-foot. The gonads and associated structures, recognizable as primordia in even the smallest specimens, are always found at the other end of the body in the part lying in the distal portion of the cyst (Fig. 1A); the reproductive system is typically posterior in the Turbellaria. It was not possible to confirm this interpretation by examining the nervous system, however, as only occasional nerve cells could be identified with any certainty. These occur laterally, perhaps as components of lateral cords, but nothing resembling cerebral ganglia could be found.

The nutrition of Acholades asteris

A. asteris lacks any trace of the typical turbellarian alimentary system and feeds via its epidermis on the collagenous and cellular components of the outer connective tissue layer of the tube-foot wall.

The epidermis and subepidermal glands. The body wall consists of a monolayered epidermis, resting on a delicate basement membrane, and a thin subepidermal musculature made up of outer circular and inner longitudinal muscles. The epidermis (Figs. 2A–D) has the same basic structure over the entire body surface but the relative numbers of cell types, their sizes and contents, and the occurrence and sizes of epidermal invaginations vary in different areas. The most profound modifications occur anteriorly where the cyst is attached to the tube-foot. Over most of the body surface, posterior to this region, the epidermis is 25–30 μm in height and composed

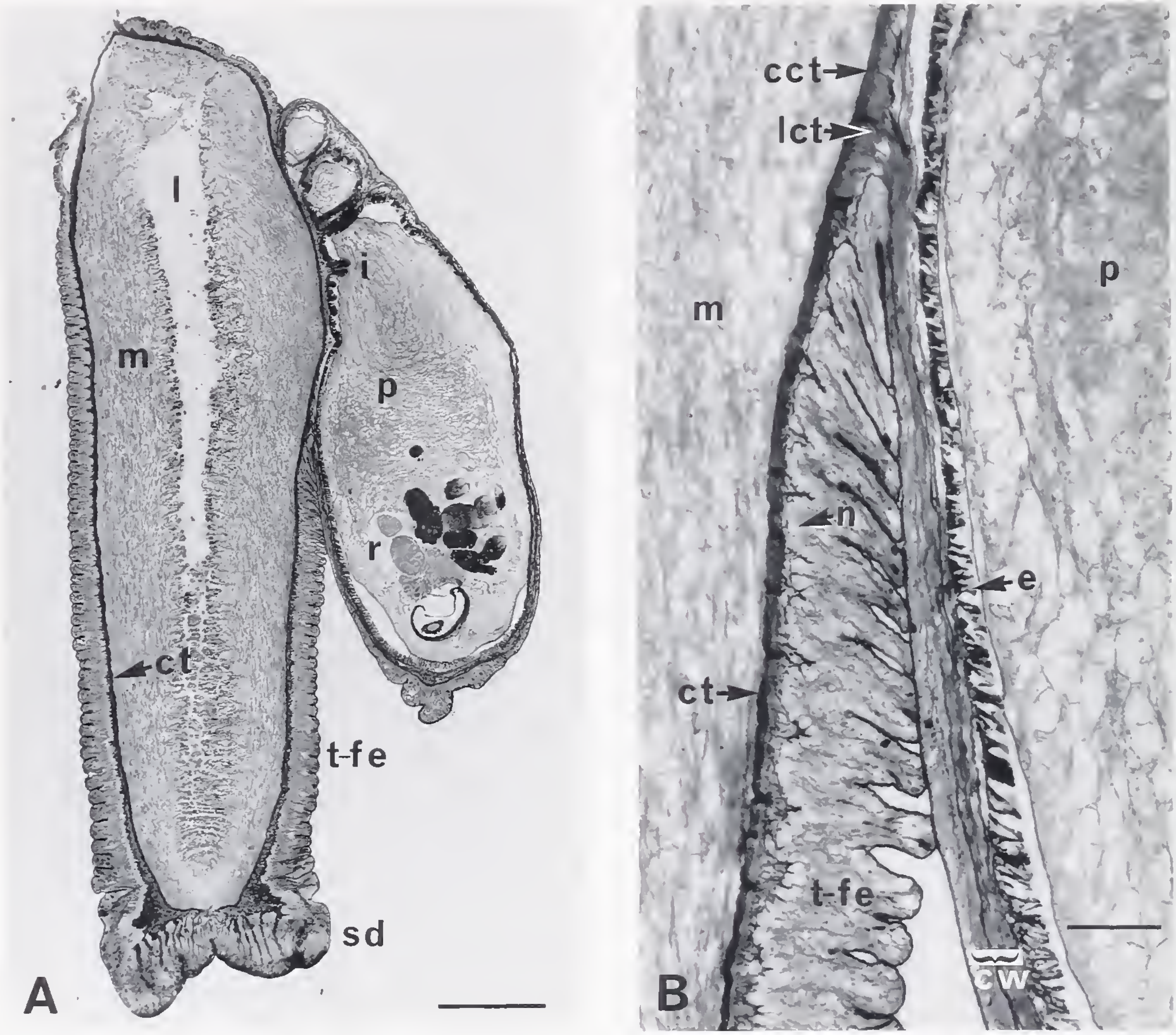

Figure 1. Longitudinal sections through a retracted ambulacral tube-foot of *Coscinasterias calamaria* with an attached cyst (right) containing a mature *Acholades asteris.* (A) An entire section showing the general anatomy of the tube-foot, cyst, and worm (section stained with alcian blue, periodic acid-Schiff and orange G; scale = 0.5 mm). (B) Detail from another section showing the structure and relationship of the tube-foot and cyst walls (stain as in (A); scale = 75 μm). cct, circular connective tissue; ct, connective tissue layers (circular and longitudinal) of tube-foot wall; cw, cyst wall; e, epidermis of *A. asteris;* i, major invaginations of *A. asteris* epidermis; l, lumen of tube-foot, lined by coelomic epithelium; lct, longitudinal connective tissue; m, muscular layer of tube-foot; n, nervous layer; p, inner compact parenchyma; r, reproductive organs of *A. asteris;* sd, sucker disc of tube-foot; t-fe, tube-foot epidermis.

mainly of ciliated columnar cells, 10–12 μm wide, with basal nuclei and various cytoplasmic inclusions similar to those to be described in the cells of the attachment area. Short microvilli occur sparsely between the cilia. Oval gland cells, 12–15 μm by 8–10 μm, occur between the basal regions of the columnar cells in a ratio of approximately 1:20 of the latter. The gland cells have basophilic inclusions, 1–3 μm in diameter, which give thermolabile positive reactions for endopeptidase when incubated in the indoxyl acetate medium for non-specific esterases at pH 4.5 in the presence of 10^{-3} *M* cysteine.

Small v-shaped invaginations, densely lined with cilia, occur along the epidermis at intervals of 30–60 μm; these may be as little as 10–15 μm deep and involve only 2–5

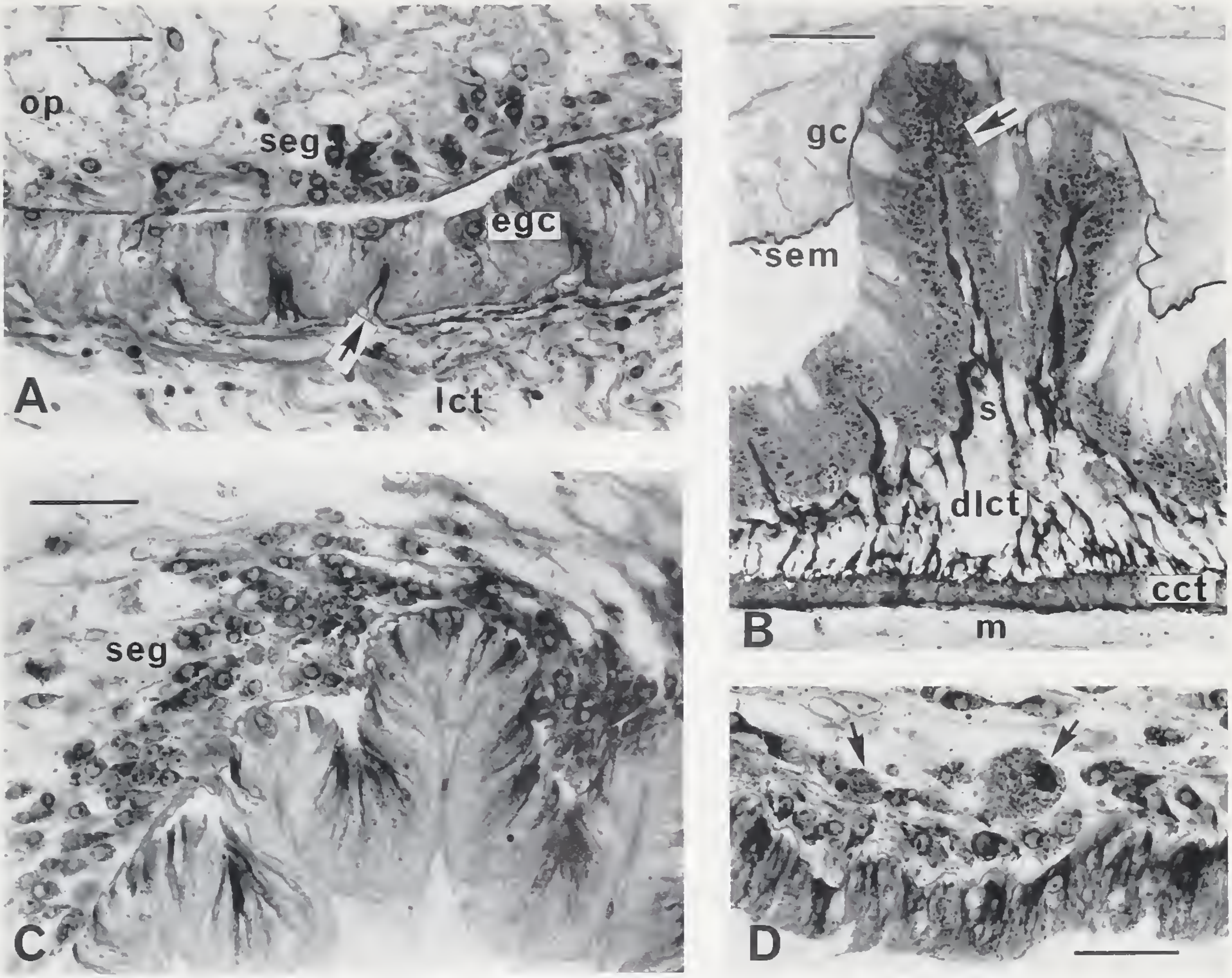

Figure 2. The epidermis and associated structures in *Acholades asteris.* (A) A portion of the epidermis in the mid-body region showing the characteristic v-shaped intercellular invaginations (arrowed) (haematoxylin and eosin; scale = 30 μm). (B) A portion of invaginated epidermis in the attachment area, showing deeply staining phagosomes (arrowed) in the distal regions of the columnar cells (alcian blue, periodic acid-Schiff and orange G; scale = 35 μm). (C) As (B) but stained to show the concentrations of subepidermal glands below the major invaginations (Giemsa; scale = 40 μm). (D) A portion of the body wall of *A. asteris* adjacent to the attachment area, showing two gametocysts of the gregarine *Monocystella* sp. (arrowed) (Giemsa; scale = 25 μm). cct, circular connective tissue of tube-foot wall; dlct, longitudinal connective tissue being digested by enzymes from *A. asteris;* egc, epidermal gland cell; gc, epidermal and subepidermal gland cells (not stained); lct, longitudinal connective tissue of cyst wall; m, muscular layer of tube-foot wall; op, outer parenchyma; s, strands of connective tissue drawn into epidermal invaginations; seg, subepidermal gland cells; sem, subepidermal musculature (contracted away from epidermis during fixation).

cells if they are at 90° to the basement membrane. When they run obliquely into the epidermis they can be more extensive and involve 5–10 cells (Fig. 2A).

Subepidermal gland cells occur at intervals in the parenchyma adjacent to the subepidermal musculature (Figs. 2A–D). The cell bodies are 30–40 μm long, basically spindle-shaped but often tapering sharply from a central expanded area containing the nucleus. They have strongly basophilic cytoplasm, with granular inclusions, and are produced at one end into long, extremely slender necks that pass outwards through the musculature and either through or between the epidermal cells to open between the bases of the cilia. The majority lie parallel to the epidermal surface so that their necks, or ducts, bend sharply as they pass outwards. The granular secretions are similar to those of the epidermal cells, being basophilic and showing strong reactions for acidic endopeptidase.

The epidermis becomes thicker anteriorly, with the height of the columnar cells rising to 40–60 μm. The number of gland cells also increases and their ratio to the columnar cells may rise to 1:4 of the latter (Fig. 2B). This thickened, highly glandular epidermis is deeply invaginated in the areas where it faces the split outer layer of the tube-foot's connective tissue, in the region where the cyst is attached to the tube-foot column (Figs. 1A, 2B, C). The invaginated epidermis is accompanied by the subepidermal musculature and glands; there are usually four or five major invaginations, one or two of which may extend into the parenchyma for up to a quarter of the diameter of the turbellarian's body. Their epidermal linings very often possess secondary intuckings of the type seen elsewhere over the body surface, but, like these, they involve only epidermal cells and not the underlying muscles. The columnar cells of both types of invagination remain fully ciliated.

The number of subepidermal glands increases markedly in this anterior region and are especially concentrated around the deep epidermal invaginations where they form a compact layer several cells thick (Fig. 2C). However, each gland cell retains its own duct which carries its secretions into the ciliated lumen of the invagination. The majority of the ducts pass through the columnar epidermal cells and any one of these may be traversed by several ducts; the secretion appears to be in membrane-bound globules 100–150 nm in diameter, so far as could be judged from the poorly fixed available material.

Digestion and uptake of host tissues. Endopeptic secretions from the epidermal and subepidermal glands attack the outer connective tissue layer of the tube-foot in the attachment region of the cyst, where it is exposed by the separation from it of its own outermost fibers and the tube-foot epidermis to form the cyst wall (Fig. 2B). Bundles of collagen fibers, connective tissue cells, and coelomocytes become progressively disorganized and are eventually reduced to an amorphous broth whose constituents, both particulate and fluid, show a blend of the staining reactions given by the unaltered tissue elsewhere in the tube-foot wall. This semi-digested broth occurs in large amounts in the attachment region and within the major epidermal invaginations facing the area attacked. It also bathes the turbellarian's entire epidermal surface and enters the various minor invaginations. Away from the attachment region, the turbellarian's epidermis is closely applied to the cyst wall so that only relatively small amounts are present. The inner face of the cyst wall (the extended outer connective tissue layer of the tube-foot wall) shows no evidence of digestive attack. Therefore, I conclude that the endopeptic secretions from the epidermal and subepidermal glands discharged away from the attachment region merely continue digestion of the broth. How this is achieved without damaging the cyst wall remains unknown.

The major epidermal invaginations often contain in their lumina strands of collagenous material in addition to the broth (Fig. 2B). These must have been drawn in by ciliary action, appropriate localized activity of the invaginated subepidermal musculature, or a combination of these.

Disruption of the longitudinal connective tissue usually extends inwards for about one half of its width. The layer is not breached, and the inner circular connective tissue, podial musculature, and coelomic epithelium are not affected. This situation was found in all 23 specimens examined, indicating that regeneration of the damaged layer keeps pace with the digestive attack. In many specimens the layer reacted by proliferating and thickening and the attachment area contained larger numbers of coelomocytes than were present in comparable areas of uninfected tube-feet.

The broth of digesting material is taken into the turbellarian's epidermal cells in pinocytotic vesicles which form between the bases of the cilia (Fig. 3A). The vesicles often contain whorled membranes and other particles, as well as more homogeneous materials; they move down into the cells and several fuse to form large phagosomes (Figs. 3A, B). The pinocytotic vesicles are just visible with the light microscope as tiny spheres staining with PAS and acid fuchsin. However, the phagosomes are prominent features of the epidermal cells, reacting strongly to PAS and acid fuchsin and lying in the distal to middle thirds of the cells (Fig. 2B). They are 1–3 μm in diameter when newly formed but decrease in size as they pass further into the cells and as digestion of their contents proceeds. Their shrinking is accompanied by progressive decrease in their response to PAS. In the basal regions of the cells their remnants become PAS-negative and stain only weakly with acid fuchsin or haematoxylin.

The same sequence of events occurs elsewhere in the epidermis, away from the attachment area, but to a much lesser extent. Phagosomes can usually be seen in the epidermis covering the middle region of the body, but within groups of cells rather than continuously. They are very infrequent posteriorly in the genital region.

Food reserves. The parenchyma filling the interior of the turbellarian's body is a syncytial tissue made up of an outer diffuse layer surrounding a compact and more heavily staining core (Fig. 1A). Glycogen is the principal food reserve. Large quantities seen as PAS-positive, amylase-labile granules occur in the inner parenchyma and

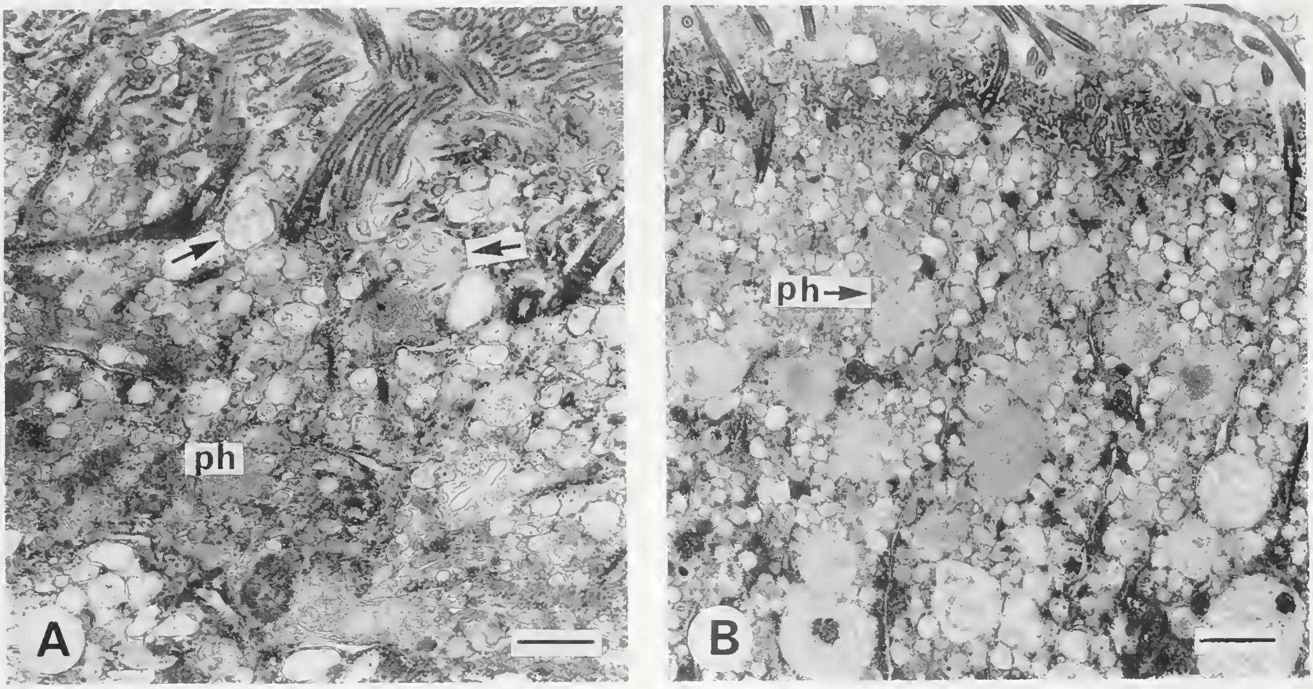

Figure 3. Transmission electron micrographs of the distal portions of epidermal cells in *Acholades asteris.* (A) Pinocytotic vesicles (arrowed) forming between the bases of the cilia (scale = 0.5 μm). (B) Pinocytotic vesicles and phagosomes formed by fusion of such vesicles packing the distal regions of five columnar epidermal cells (scale = 1 μm). ph, phagosome.

smaller amounts in the ovaries and vitellaria. Only small amounts of lipids are laid down. These occur sparingly in the inner parenchyma and in somewhat larger quantities in the ovaries and vitellaria, as osmiophilic globules 1–2 μm in diameter.

Hyperparasitic protozoa

Six of the 23 *Acholades asteris* examined were infected with an aseptate gregarine protozoan identified as an undescribed species of *Monocystella.* The gregarines occur in the anterior region of the turbellarian, in the outer parenchyma and epidermis, and are associated particularly with the large epidermal invaginations. Young trophozoites from the parenchyma invade the epidermis where they grow to a length of 40–50 μm. When fully grown they often show a more lightly staining region anterior to the nucleus, especially with Giemsa or Azur II. They apparently migrate back to the outer parenchyma, as mature gamonts, since syzygy was only seen here and never in the epidermis. Syzygy is followed by formation of isogamous gametocysts that often contain residual cytoplasm (Fig. 2D). The remainder of the life cycle is unknown. Sporocysts, oocysts, and sporozoites were not found and no stages were seen outside the turbellarian in the attachment area, cyst wall, or tube-foot.

Discussion

The principal feature of interest emerging from these observations is the unequivocal demonstration that in this unusual turbellarian, *Acholades asteris,* the epidermis and subepidermal structures function as a complete alimentary system through which the animal obtains all its food. Supplementary uptake of organic nutrients via the epidermis occurs in a wide range of free-living aquatic invertebrates (see reviews by Stephens, 1972; Jørgensen, 1976; Stewart, 1979) and in entoparasites such as some digenetic trematodes (Smyth and Halton, 1983). However, in these instances the substances taken in are already dissolved in the surrounding medium. This is also the case in fecampiid turbellarians living in hemocoels, and in cestodes. Admittedly, in the latter there is evidence that phosphatases and nonspecific esterases on the outer surfaces of the apical plasma membrane convert non-transportable organic phosphate esters into transportable organic bases and phosphate (Lumsden and Specian, 1980; Threadgold, 1984) but this is not the same as the situation found in *A. asteris.* Here, the flatworm secretes digestive enzymes from modified epidermal and subepidermal glands which attack organized components of the surrounding host tissue, degrading them into particulate or soluble substances. These are taken into the epidermal cells by pinocytosis; digestion is completed intracellularly. The entire process is directly comparable to that occurring in the gut of most other turbellarians, whether free-living or symbiotic, after ingestion of prey organisms or host tissues by muscular pharyngeal feeding mechanisms (Jennings, 1977, 1985; Garcia-Corrales and Gamo, 1988). Particularly noteworthy is the occurrence in *A. asteris* of a large intracellular component in its digestive physiology, exactly as in other turbellarians, even though the digestive tissue is ectodermal epidermis and not entodermal gastrodermis.

The columnar cells in which digestion is completed remain densely ciliated despite the large number of pinocytotic vesicles constantly forming in their distal surfaces. The cilia are probably essential for circulation and mixing of the enzymes secreted from and through the epidermis, and for moving the semi-digested broth away from the attachment area and over the general body surface for uptake elsewhere. This retention of cilia on cell surfaces concerned with uptake of particulate as well as soluble materials is unusual but has been reported also for nemertean worms. In these it is gastrodermal cells that are involved and materials are taken up by phagocytosis, with large phagosomes forming directly and not from fusion of pinocytotic vesicles (Jennings, 1969).

The minor epidermal invaginations seen in *A. asteris* can be regarded, simply, as anatomical adaptations increasing the surface area available for enzyme-substrate activities and nutrient uptake, similar to the folds and villi-like structures found in most alimentary systems. This view can also be taken of the major invaginations but these are very specialized structures with hypertrophied cells and greatly increased numbers of gland cells; they are accompanied by invagination of the underlying musculature and proliferation of the associated subepidermal glands. They function, in fact, as miniature guts, drawing into their lumina strands of host tissue for digestion (Fig. 2B). If the orientation of *A. asteris* within the cyst has been interpreted correctly then these major invaginations, always sited together and in the same area, are seen to be anterior and sub-terminal. They are thus in the same relative position as the stomodaeal invaginations in the embryos of other turbellarians, which develop into the mouth and pharyngeal region, and certainly resemble these when seen in section if the proliferation of their glandular components is ignored. Therefore, they may represent an alimentary system that has not developed along orthodox lines and which is supplemented by concomitant modifications of the epidermis over the rest of the body.

Proliferation of sub-epidermal glands, but without regionalization and anatomical modifications of the epidermis, is a consistent feature of other parasitic turbellarians that lack normal alimentary systems, such as *Fecampia*, *Glanduloderma*, and *Kronborgia* (Caullery and Mesnil, 1903; Jägersten, 1942; Shinn and Christensen, 1985). Their secretions, and those of epidermal glands, are concerned with cocoon formation and it is not known if they also play a part in nutrition.

A. asteris may use organic nutrients in addition to those obtained by its extra-corporeal digestion of host tissues. Epidermal uptake of dissolved organic materials such as amino acids is well documented for echinoderms and especially asteroids (Ferguson, 1971; Péquignat, 1972; Stephens *et al.*, 1978; Bamford, 1982). The tube-feet form an enormous surface area for this and thus *A. asteris* is well placed for carrier-mediated abstraction of any nutrients passing inwards from the exterior. Indeed, since the cyst wall is simply an extension of the two outer components of the tube-foot wall, with the turbellarian in effect taking the place of the inner components, nutrient absorption by the cyst wall epidermis could contribute significantly to the parasite's nutrition. Further potential sources of nutrients are the migratory coelomocytes visiting the tube-foot wall, whose numbers increase in the presence of *A. asteris*, and the hemal strands adjacent to the bases of the tube-feet. The coelomocytes and hemal system are both involved in nutrient translocation within the starfish body (Ferguson, 1982).

The favorable surface area-volume ratio of the cyst, comparable to that of the tube-foot, and the thinness of the cyst wall, will facilitate gaseous exchange in *A. asteris*. The flatworm has not evolved a specific respiratory pigment such as the hemoglobins found in other entosymbiotic turbellarians that live deeper within their respective hosts (Jennings and Cannon, 1987; Jennings, 1988). The emphasis on glycogen in the food reserves probably has no respiratory significance. It is a feature common to many entosymbiotes and is likely to be related to the reproductive physiology as discussed in detail elsewhere (Jennings and Calow, 1975).

It is clear, then, that the asteroid tube-foot, unlikely though it might seem at first sight, is a favorable habitat for *A. asteris*. The turbellarian is well adapted to it physiologically and also behaviorally. The unknown larval stage must show a considerable degree of site selection when it enters the tube-foot wall, settling within the outer connective tissue layer at a point well away from the podial nerve strand. Preservation of the strand and of the circular connective tissue layer and musculature, vital for the functioning of the tube-foot (Smith, 1946, 1947; Hyman, 1955), allows the tube-foot to remain alive and thus also preserves the cyst. The feeding activities are in balance with the host's regenerative capacities and do not involve the cyst wall which can continue to grow to accommodate the growing parasite. Cysts were not found in the sucker-disc region but were described from there by Hickman and Olsen (1955). Their presence presumably restricts the adhesive function of the tube-feet but will not be lethal to these if the cyst is formed similarly to those occurring on the podial columns.

The occurrence of the gregarine *Monocystella* sp. in *A. asteris* is of particular interest in view of its location. *Monocystella* spp. are typically parasites of turbellarian alimentary systems (Levine, 1977). For example, the hyperparasitic *M. epibatis* has recently been described from the rhabdocoel *Pterastericola vivipara,* which is itself entoparasitic in the pyloric ceca of the Crown of Thorns starfish *Acanthaster planci* on the Great Barrier Reef (Cannon and Jennings, 1988). *M. epibatis* trophozoites and gamonts live in the gastrodermis and intestinal lumen of their host. The similarly hyperparasitic *Monocystella* sp. found in *A. asteris* retains this association with turbellarian digestive regions, its trophozoites and gamonts having colonized the epidermis and subepidermal parenchyma.

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Marine Models in Biomedical Research

A Report from the Marine Life Resources Workshop at the
Duke University Marine Laboratory, Beaufort, North Carolina
May 1988

Preface. In 1985, the National Institutes of Health (NIH) sponsored an evaluation by the National Academy of Science of modelling in biomedical research. The report of the evaluation, "Models for Biomedical Research: A New Perspective," recommended that NIH encourage interest in nonmammalian systems and develop such systems as biomedical research models. In response to this recommendation, a workshop was organized expressly to consider the contributions to biomedical research that marine and freshwater organisms make, and may make, and to assess the need for resource sites that would provide these animals to the research community.

The workshop comprised an *ad hoc* group of researchers from various institutions and representatives from some federal agencies (see Appendix). The group met at the Duke University Marine Laboratory, Beaufort, North Carolina, in May 1988. The following report contains recommendations made by workshop participants. It is designed to help the NIH's Division of Research Resources (DRR) develop and formulate plans for future support of marine life resources through its recently established Biological Models and Materials Resources Section.

Although the title of this report refers to marine models, it became apparent, as the organization of the workshop unfolded, that freshwater organisms should also be considered. Indeed, the term "marine" in this report will often include freshwater organisms. Also, species were not limited to invertebrates, but research topics were limited to nonmammalian freshwater and marine animals.

Reprint requests: Director's Office, Marine Biological Laboratory, Woods Hole, MA 02543.

Recommendations

The participants in the workshop made the following recommendations:

- The value of nonmammalian models to the biomedical sciences is manifest. Therefore, we recommend that the NIH and other appropriate federal agencies increase their support of fundamental studies involving nonmammalian marine and freshwater animals.
- We recommend that the NIH fund and encourage the development and improvement of facilities that provide nonmammalian marine or freshwater species as resources to the biomedical community. We need to solve technical problems, including water quality, temperature, tanks, and nutrition.
- We recommend that individuals in the review process at NIH, such as the review committee members and the executive secretaries, be better educated about the importance of comparative studies.
- We strongly urge that study section panels reviewing proposals on facilities, and on research involving the use of marine animal models for biomedical research, be composed of experts in these fields. In any event, these panels should not be limited to individuals who are expert at operating mammalian facilities.
- Congress and the public must be taught that research on marine organisms is an important component of biomedical science. This activity should be performed by the research community.
- We must re-emphasize a recommendation made in the National Academy of Science's report, "Models for Biomedical Research: A New Perspective." Proposals for the study of invertebrates and lower verte-

Table I

A selection of marine model systems for biomedical research

Model	Area of study	Advantages
Coelenterata		
jellyfish	Axonal and synaptic physiology	Unique structure and organization; accessibility
Polychaeta		
fanworm	n-hexane neuropathy	Accessible, large syncytial axon
Mollusca		
bivalve	Hemic neoplasia	Large number of individuals, possible intermediate of human disease
Cephalopoda		
octopus	Learning and memory	Well-developed CNS, learning and memory confined to one part of CNS
Merostomata		
Limulus	Macromolecular assembly; hemocyanin	Ribosome-sized molecules can be isolated in large quantity, recombined and positioned in the intact oligomer
	Vision	Large, accessible photoreceptors in a simple eye
Crustacea		
barnacles	Muscle research	Muscle cells are very large
Callinectes	Kidney function	Tissue is a single cell thick, and large
Homarus	Hormonal control of behavior	Individual neurons can be identified and isolated
Panulirus	Olfaction	Receptor cells amenable to biochemistry and electrophysiology
Echinodermata		
sea urchin	Cell and developmental phenomena	Large quantity, can obtain synchronous development
Tunicata	Allorecognition	Genetically identical blastzooids, displays colony specificity
Osteichthyes		
Poeciliopsis	Carcinogenesis—host determinants	Stable subpopulations of members of natural population
Teleostei	Essential and toxic metal metabolism	Large basal level of liver metallothionein for metabolic studies
flounder	Solute transport across membranes	Accessible organs and cells
	Kidney function	Simple (proximal) tubule, easy to isolate and maintain
trout	Carcinogenesis	Sensitivity; large studies with genetically diverse individuals
Elasmobranch	Development and reproductive biology	Novel reproductive physiology

brates should be regarded as having the same potential relevance to biomedical research as proposals for studies using higher organisms.

- We recommend the establishment of databases for marine models and for core facilities that handle or culture marine organisms. Researchers could then obtain necessary information on modelling, culturing, and supplying of organisms.

Introduction

Aquatic models

Rodents and other mammalian species are perceived as the models of choice for biomedical studies. However, participants at this workshop argued convincingly that other living systems also have much to contribute to such research. The reasons are as diverse as the organisms themselves. Some currently used and potential aquatic model systems were discussed at the meeting, and participants agreed that the distinction between existing and developing models is often blurred. Their applicability to important biomedical questions are tabulated above (Table I). This table represents only a fraction of the models that are currently being used or developed, and reflects only the areas of research of the investigators at the workshop. However, even this limited list suggests the broad range of biomedically related research topics that can be investigated using aquatic organisms. These examples underscore how nonmammalian organisms expand biomedical research possibilities. Participants also discussed some physiologically important molecules that have been isolated and characterized from marine organisms (see Table II).

Table II

Some physiologically important molecules that have been isolated and characterized from marine organisms

Molecule	Organism	Use for research
Aequorin	Marine jellyfish	Study actions of ionic calcium in many biological processes
SCPs and other neuropeptides	Molluscs	Study physiological roles of these molecules in vertebrates
Polyphenolic protein	Mussels	Adhesive protein with possible application as medical adhesive
Manoalide	Marine sponge	Inhibitor of arachidonic acid cascade enzymes—anti-inflammatory agent
Cytochrome c oxidase	Shark	Mitochondrial oxidase half the molecular weight of human enzyme

Facilities

Many institutions operate facilities that provide aquatic organisms to their own investigators and to researchers from other institutions. Some of these facilities (*i.e.*, those of workshop participants) are listed in Table III. Of course, there are many other resource centers throughout the United States that provide aquatic organisms to researchers. This sampling of facilities indicates the wide range and diversity of organisms available.

Current Support of Marine Research and Resources by Federal Agencies

NIH

Dr. Jerry Robinson of the NIH's National Institute of Environmental Health Science (NIEHS), explained that NIEHS is a resource and focal point of research on the adverse effects of environmental chemicals and other factors on human health. In pursuit of these objectives, NIEHS fosters and encourages the use of aquatic organisms as experimental subjects or models at five centers, which it supports under the Marine and Freshwater Biomedical Sciences Centers Program.

Dr. James Willett of the NIH's Division of Research Resources (DRR) discussed the programs of the Biological Models and Materials Resources Section within the Animal Resources Branch of DRR. The Section promotes responses to the recommendations of the National Academy of Science's report described above. In particular, the Section provides for the development and support of cell systems, lower organisms, and nonbiological systems as models for biomedical research. It also provides critical biological materials for the research community.

NSF

Dr. James Edwards of the National Science Foundation (NSF) discussed his division's support of resources for the distribution and maintenance of living organism stocks to biomedical researchers. This program does not currently support marine-oriented stock centers. However, there is no programmatic impediment preventing marine stock centers from applying. Another program within this division concerns Equipment and Facilities for Research at Biological Field Stations and Marine Laboratories. A competitive program exists to improve research facilities and equipment at such sites.

Dr. Joan R. Mitchell indicated that the NSF's Biological Oceanography Program focuses on biochemistry, chemical ecology, and marine biotechnology, and is interested in proposals that integrate the tools of molecular biology into ocean sciences. Proposals for the culture of marine organisms useful in research and biotechnology are encouraged.

ONR

Dr. Michael Marron of the Biological Sciences Division, Office of Naval Research (ONR), reported that his division supports basic biological research in the Molecular Biology and Systems Biology programs. The objective of the Molecular Biology program is to extend our basic understanding of the principles of biological processes at the molecular level with an emphasis on marine microorganisms. The program is focused on molecular aspects of marine biology and microbiology, and on determining the principles of structure and function of biological molecules. It also funds courses at marine stations. The Systems Biology program supports research on the cellular responses of biological systems to environments of special concern to the Navy.

NOAA

Ms. Anne Studholme of the National Oceanic and Atmospheric Administration (NOAA) is involved with the National Marine Fisheries Service's Northeast Fisheries Center, which is one of four centers administered by the

NMFS. She explained the centers' programs, which assess the effects of environmental parameters on marine fish.

Conclusions and recommendations

Although some federal agencies (including ones not represented here) support marine facilities and biomedical research using marine organisms, funding for these activities must be improved substantially. Only a fraction of the currently funded grants at the NIH involve the use of nonmammalian animals.

We strongly recommend that the NIH and other appropriate federal agencies increase their support of research that involves nonmammalian marine and freshwater animals.

Resource Potentials for the Mariculture and Aquaculture of Organisms of Biomedical Interest

Many factors must be considered in developing marine resources as biomedical models, including the facilities and expertise necessary to maintain animals under intensive, controlled, and large-scale culture conditions that also permit growth and reproduction. A major problem in the use of certain marine organisms for biomedical research is that they have restricted breeding seasons. Thus there are periods during the year when they are unavailable in large quantities for research. Therefore, researchers have developed methods of culturing some of these organisms in the laboratory. As a result, all stages of an organism's life cycle may be available for use at all times.

As noted by Dr. Judith P. Grassle (Marine Biological Laboratory), the technology for maintaining marine species under stringent environmental requirements has advanced considerably in recent years. For example, large public aquaria can now maintain marine organisms in recirculating seawater systems. Such advancements have increased the number of species that can be reared successfully in laboratory culture, and have thus increased the potential for providing many species to researchers year-round.

Dr. A. O. Dennis Willows (Friday Harbor Laboratories) explained that several species of nudibranchs (sea slugs), such as *Hermissenda* and *Tritonia*, contain clearly identifiable brain cells that are the largest known in the animal kingdom. These cells have been used to answer general questions about neurons; *i.e.*, the existence and roles of ion channels in neuronal membranes. As discussed by Dr. Alan Kuzirian (Marine Biological Laboratory), the laboratory culture of some of these mol-

Table III

Some facilities supplying aquatic organisms to researchers

Site	Facilities
Battelle Marine Sciences Laboratory, Sequim, WA	Culture of bivalve molluscs and salmonid fish for biomedical research and environmental testing
California Institute of Technology, Pasadena, CA	Sea urchin culture, development of inbred and transgenic animals
Friday Harbor Laboratories, University of Washington, Friday Harbor, WA	Maintenance of diverse marine organisms using high-quality seawater
Hopkins Marine Station, Stanford University, Pacific Grove, CA	Culture of the tunicate *Botryllus*. Maintenance of many diverse marine organisms
Marine and Freshwater Biomedical Center, Duke University Marine Laboratory, Beaufort, NC	Sea urchins, *Limulus*, and diverse organisms supplied. Octopus and other other marine organisms maintained for marine research
Marine and Freshwater Biomedical Center, Oregon State University, Corvallis, OR	Complete rainbow trout hatchery and cancer laboratory
Marine and Freshwater Biomedical Center, University of Wisconsin, Milwaukee, WI	Facility patterned after mammalian quarters to hold and use diverse aquatic organisms for biomedical research
Marine Biological Laboratory, Woods Hole, MA	Maintenance of diverse marine organisms with different environmental requirements
Marine Biomedical Institute, University of Texas, Galveston, TX	Squid and octopus mariculture in recirculating artificial seawater
Mote Marine Laboratory, Sarasota, FL	Maintenance of diverse marine organisms. Culture of clearnose skates
Mt. Desert Island Biological Laboratory, Salisbury Cove, ME	Collection and maintenance of sharks and skates in seawater
New England Aquarium, Boston, MA	Lobster culture, including all stages of development
University of Maine, Orono, ME	Culture of fanworm, *Myxicola*
The Whitney Laboratory, University of Florida, St. Augustine, FL	Various marine species maintained in high-quality seawater; controlled temperature and light
Woods Hole Oceanographic Institution, Woods Hole, MA	Marine culture center for diverse organisms using high-quality seawater

luscs has made them particularly advantageous biomedical research models. At least 10 opisthobranch species have been reared through their complete life cycle in the laboratory. In particular, *Hermissenda* and other small nudibranchs have the advantage over other opisthobranchs by requiring less space, food, and maintenance. They also have shorter life cycles. *Aplysia* has been reared successfully in artificial seawater (AWS), and the same should soon be possible for *Hermissenda*, which is currently being cultured using natural seawater. Their ASW compatibility makes these animals available to land-locked research facilities.

Cephalopods have played an important role in biomedical research, especially in neurobiology. However, squid are highly active, predatory animals that are easily injured during collection, transport, handling, and maintenance. The feasibility of mariculture of squid, octopus, and cuttlefish was demonstrated by Dr. Roger T. Hanlon (Marine Biomedical Institute). He evaluated nine species of squid, fourteen species of octopus, and two species of cuttlefish, and chose the prime candidate of each group to culture through its life cycle (usually 6–12 months) in recirculating, high-quality, artificial seawater. A major impediment in culturing these cephalopods is the high cost of the live food needed to maintain or rear these carnivores. A program is currently underway to develop an artificial diet for octopus, which will be expanded to squid and cuttlefish.

The marine polychaete *Myxicola* is a subtidal cold water annelid that is gaining popularity in neuroscience research because of its large, accessible syncytial axon. To date, the supply of these animals for biomedical research has depended upon hand-collected specimens. Dr. David Dean (University of Maine, Orono) has cultured these fanworms in unsupplemented seawater at temperatures between 8–12°C. He has successfully maintained the worms in the laboratory in excess of three years with negligible mortality. Progeny have been raised through the second generation. The potential for the development of genetically identical clones makes this organism attractive for researchers. The organisms can be shipped easily in plastic bags filled with seawater.

The sea urchin is an ideal system for studies on cell and developmental phenomena since gametes are easily obtained in large quantity, and synchronous cycles of mitosis and cytokinesis can be accomplished by simple mixing of sperm with eggs. Currently, sea urchins are harvested from the natural environment. Dr. David Epel (Hopkins Marine Station) indicated that there is very little farming or maintenance of sea urchins, and researchers must either do their own collecting or depend on commercial suppliers.

The sea urchins most used in the laboratory are *Strongylocentrotus purpuratus* and *Lytechinus pictus*. However, these species have restricted breeding seasons, so for one or two months each year, no animals are available in large quantities. A potential solution to this problem has come from Dr. Eric Davidson's laboratory at the California Institute of Technology, where a culture system for maintaining gravid animals year-round has been developed. Dr. Frank J. Calzone (California Institute of Technology) indicated that over 3000 animals per year are provided to researchers. Moreover, inbred colonies and transgenic animals are being developed. However, the system would have to be expanded to make the sea urchin available to many researchers. Inbred lines represent an invaluable resource for molecular genetic analyses, but culturing is also critical since future stocks of sea urchins are threatened by an unregulated sea urchin fishery.

The lobster is currently a valuable model for studies on the molecular basis of the ontogeny of social behavior including violent or aggressive behavior, for examination of neural influences on muscle fiber differentiation, and for studies of the hormonal control of reproduction. A year-round facility for holding egg-bearing lobsters, for hatching larvae and for raising the larvae to juvenile animals has been constructed at the New England Aquarium. Dr. Edward A. Kravitz (Harvard) discussed the culturing procedures used at the facility. On a six-week schedule, individual egg-bearing lobsters are taken from storage tanks where they are kept at a low temperature (under 10°C) and placed into hatching tanks at about 18°C. This temperature shift dramatically increases the rate of development of the 10,000 to 100,000 embryos attached externally to the swimmerets of the mother. Larvae hatch and go through a series of developmental stages. The larvae and the juvenile animals are kept in special tanks, depending on their stage of development. Up to several hundred juvenile animals can be housed for experiments. The rearing facility can produce animals whose entire experimental history is known, and lobsters are available for behavioral and other experimental studies.

Dr. Dale F. Leavitt [Woods Hole Oceanographic Institution (WHOI)] discussed the Environmental Systems Laboratory (ESL) at WHOI. The laboratory was built as a pilot scale bivalve culture facility, and is now a general marine culture center. An elaborate, redundant system has been constructed to provide the facility with unlimited quantities of very high quality seawater, and to ensure an uninterrupted seawater flow. Unique procedures have been developed to circumvent potential limitations for culture; *i.e.*, cod liver oil feed supplement was formulated and fed to larval lobster to increase survivability during metamorphic molt between planktonic stages III and IV. Work currently underway at WHOI exemplifies the types of phenomena that show high potential as biomedical models. For example, one model is a hematopoietic neoplasia in the clam, *Mya arenaria*, which may prove similar to some mammalian leukemias.

The Marine Biological Laboratory (MBL) in Woods Hole supplies nearly 100,000 marine organisms per year to its own researchers and those of other institutions. As discussed by Dr. Grassle, organisms such as squid, surf clams, horseshoe crabs, fish, lobster, and sea slugs and snails are used at, and shipped from, the MBL. However, the facilities for performing this task fall short of what is necessary and technologically feasible. Thus, a marine resources center is being designed to maintain diverse marine species, with different environmental requirements, in good health and reproductive condition.

Plans at the MBL include the addition of tanks with a flow-through capacity of approximately 32,000 gallons of ambient seawater so that large numbers of animals can be maintained in lower densities and in better condition. A recirculating seawater system is planned, but will be demanding in its maintenance costs. Small, ancillary recirculating systems will enable researchers to conduct experiments on marine animals using labelled compounds and pollutants; neither type of chemical can be released into the environment.

Biomedical research in toxicology is being performed at the University of Wisconsin-Milwaukee Marine and Freshwater Biomedical (MFB) Core Center using aquatic models to study mechanistic questions related to human environmental health problems. Center Director Dr. David Petering outlined the plans for renovating the Center's animal quarters into a facility for holding and experimenting with aquatic organisms. The experimentation rooms will have environmental controls and flexibility comparable to high quality research space for mammalian organisms. Currently, the Center is using the tadpole to model the effects of cadmium and lead on the immune system which develops similarly to that in mammals. The microorganism, *Euglena gracilis*, is also being used to study the nature of metal-metal interactions in heavy metal toxicity. A group of personnel at the new facility will work with scientists who are not skilled in the use of aquatic organisms. Thus, the facility will be accessible to investigators interested in comparative studies.

At the Mt. Desert Island Biological Laboratory (MDIBL), marine and freshwater species, especially fishes, are used in biomedical research. As explained by MDIBL Director Dr. David H. Evans, the laboratory focuses on the mechanisms by which solutes are transported across the membranes of various cells and epithelia. Current experimental species include the dogfish shark, winter flounder, and little skate. Researchers at the MDIBL come from both clinical and basic science backgrounds, providing a broad collaborative approach to questions of fundamental biomedical interest.

Conclusions

Educational and research institutions alike are currently dependent on wild populations for their specimens. The genetics, reproductive biology, diseases and nutrition of many of these animals remains largely unknown. However, researchers are attempting to switch from relying on the capture of marine organisms to culturing them. The reasons include: (1) many organisms cannot be collected at certain times of the year due to breeding or location of the animals; (2) landlocked laboratories have to rely on others, whether an institution or commercial company, for their supply of animals; (3) depletion of animals from their natural environment may follow overharvesting, vulnerability to environmental spills, or natural disasters; and (4) culturing of genetically defined laboratory-reared organisms enables the researcher to investigate developmental stages of certain animals in a controlled environment.

Recommendations

Various federal agencies have ongoing programs in support of marine facilities, but this type of funding is very limited. The ability to culture some marine organisms has been demonstrated, and if federal support is forthcoming, these organisms would be available to many researchers. Thus, we strongly recommended that:

- Substantial new funding be made available for the support of research facilities and equipment.
- The NIH encourage marine laboratories and other institutions to apply for funds for core support for aquatic animal facilities *without* restricted targets such as environmental health, toxicology, etc. Support will be needed for all aspects of a facility, including equipment, technical support, animal feeds, and vehicles and boats.
- The NIH make funds available for facility improvements for the laboratory culturing of aquatic organisms. Some of these resources are currently under development, and continued support will be required to accomplish institutional goals.

Marine Species as Existing Model Systems

Marine and freshwater organisms provide a number of experimental and scientific advantages as biomedical models; *e.g.*, the large number of animals, tissues, cells, or gametes that can be made available; the simplicity and accessibility of some organs and physiological systems, such as the nervous system in certain species; and the ease of experimental manipulation.

Dr. Bruce A. Fowler (University of Maryland) stressed that the normal biology of species must be respected in using them as models for biological research. Many species such as oysters undergo marked physiological changes during their reproductive cycles, with resultant changes in biochemical parameters. For instance, the massive influx of copper into metallothionein in oysters during the summer reproductive phase greatly alters the normal behavior of these proteins. This also occurs in crustaceans during the molt cycle. The stress effects of field collection procedures and aquarium adaptations should also be examined since some species may undergo altered biochemical behaviors due to these practices.

The contributions of marine and freshwater nonmammalian systems to biomedical research have been diverse and important, but only a few currently used models are discussed here.

Fish

Nonmammalian vertebrates, such as fishes, have been used in diverse studies including: pancreatic insulin secretion, hemoglobin function, ionic channels in endocrine cells, mechanisms of injury to gametogenesis by toxic substances, and cytoskeletal proteins of the retina. Also, fish have many advantages as biomedical models; *e.g.*, the conditions for maintaining and culturing many species are well understood.

Dr. Jerry Hendricks (Oregon State) uses rainbow trout as a model for carcinogenesis. The trout has several advantages in this type of investigation. Trout have a high sensitivity to carcinogens; *e.g.*, microinjection of 0.5 ng aflatoxin per trout embryo can lead to liver cancer. Thus, one can study carcinogenicity of compounds at a very low doses. Trout respond to mammalian carcinogens. Other advantages of the trout in these investigations are: the small size of the animal; the low rearing cost; the option to examine a single phase of the life cycle for carcinogenesis; the ability to perform an experiment with a large number (4000–8000) of offspring from one individual and to examine all stages of the life cycle.

The importance of heredity *versus* environment in tumor biology is difficult to assess. In freely interbreeding populations, the continuous reshuffling of genes makes it impossible to identify genotypes with high or low susceptibility to carcinogenesis. Dr. R. Jack Schultz (University of Connecticut) is using the freshwater viviparous fish of the genus *Poeciliopsis* to assess carcinogenesis on wild populations. The unique property of this model lies in the clonal form of reproduction in some of these fishes. Interspecific hybrids between *P. monacha* and *P. lucida* occur in nature as all-female "species" that produce only female offspring and transmit the *P. monacha* genome intact as a single linkage group. The susceptibility to induction of liver tumors can be compared in inbred strains of the two fishes and in hybrid clones. Using these fishes, the response to water-borne carcinogens can be assessed in genotypically diverse populations.

Mammals contain low levels of metallothionein (MT) in the liver, and it is difficult to analyze. By contrast, many freshwater fishes have very large concentrations of basal hepatic Zn, Cu-MT. Dr. Petering explained that one can probe the behavior of this protein in a key tissue, and investigate the roles of MT and related metal-binding proteins in the metabolism of Zn, Cu, Cd, and organic electrophiles. There is enough of the native, uninduced protein in some fish with large livers to do NMR studies on the 113-Cd-substituted Zn, Cu-MT, a key mixed metal MT.

In mammals, the functional unit of the kidney is the nephron, which is composed of a mechanical filter, the glomerulus, and a tubule. However, analysis of renal tubular function is complicated since the tubule is anatomically and functionally complex. As indicated by Dr. John Pritchard (NIEHS, NIH), marine models in renal physiology have been used for 60 years. Early experiments on the goosefish demonstrated that tubular secretion was a fundamental process in urine formation. More recently, renal tubules from teleost fish have been used for *in vitro* studies of renal transport processes and the mechanism of cell injury and death following toxic insult. The tubules of the marine teleost kidney are composed predominately of proximal tubule which, in mammals, is primarily responsible for secretion and reabsorption of organic solutes. These tubules from the fish can be isolated intact and are viable for long periods of time.

Estuarine animals can be used as models to investigate basic cellular physiology in, by mammalian standards, unusual conditions. Thus, the roles of enzymes in mediating cellular acclimation and tolerance can be investigated. As explained by Dr. Kennedy T. Paynter (Johns Hopkins University), estuarine animals endure large variations in many environmental conditions including temperature, salinity, pH, and oxygen concentration, and many of these animals have evolved cellular mechanisms that allow them to tolerate these extremes. Genetically, many estuarine species have relatively high numbers of polymorphic loci, and genetic clines have been demonstrated in various geographic regions. For example, a lactate dehydrogenase (Ldh) cline along a temperature/seasonal gradient exists in *Fundulus heteroclitus* along the northeast coast of North America. Dr. Dennis

Powers' laboratory (Johns Hopkins University) has demonstrated that the Ldh genotype can be associated with both hemoglobin-oxygen affinity in red cells of adults, and hatching time of developing embryos. Lactate dehydrogenase, an equilibrium enzyme, seems to mediate developmental rates in this fish.

Molluscs

Various marine molluscs have been used for more than 25 years as model systems for studying associative learning and memory, including habituation and sensitization, and behavior. Among the most widely used gastropod species are *Aplysia, Hermissenda, Pleurobranchaea,* and *Tritonia*. The cephalopod molluscs, the most highly evolved molluscan group, are renowned not only for their well-developed central nervous system (CNS) and sense organs, but also for their behavioral repetoire which is vertebrate-like in complexity, and their closed circulatory system which is unique among marine invertebrates. Its CNS contains about 10^8 nerve cells and it is capable of learning complex conditioning paradigms involving decisions. Also, learning and memory in the octopus are almost completely confined to the supraesophageal part of the CNS. Thus, octopus present important advantages as an experimental animal for studies of learning and memory (L&M).

Dr. J. David Robertson (Duke University) has used *Octopus vulgaris* as an experimental animal to test a hypothesis about the cellular basis of L&M. It has long been thought that L&M involves a rearrangement of neurites in learning neuropils, and there is evidence that synapses increase and decrease in numbers in specific cortical regions with use and disuse. The primary event is thought to be activation of protein kinases followed by phosphorylation of ion channels and activation of molecular switches. Dr. Robertson injected cytochalasin B (CB) into the lobes of the octopus brain to test the hypothesis that the primary event in L&M is the extension of filopodia analogous to those seen in nerve growth cones in tissue culture. CB blocked the ability to learn a new paradigm without interfering with recall of an established memory. Electron micrographs of the learning neuropils of the lobe showed filopial extensions.

Sea urchin

Dr. Epel discussed the sea urchin as a model for developmental studies. Since the turn of the century, the sea urchin embryo has been used to establish the chromosome theory of hereditary, the description of centrosomes, parthenogenesis, and fertilization. Work within the last 30 years established such important phenomena as stable mRNA and translational control, isolation and characterization of the mitotic apparatus, and the realization that the major structural proteins of the mitotic apparatus are microtubules. Sea urchin studies provided the first evidence of actin in non-muscle cells.

Currently, studies with sea urchins are important for understanding fertilization, cell division, and gastrulation. Many cell biological phenomena such as exocytosis, endocytosis, cell surface receptors, and the control of cell growth have involved studies with sea urchins. Also, sea urchins have been used in studies on the molecular basis of speciation and on evolutionary aspects of development.

Recent studies have demonstrated that the flagellar apparatus of the sea urchin sperm obtains its energy from a unique phosphocreatine cycle. A similar phenomena is involved in heart muscle and may be involved in providing energy to all flagella and cilia. The sea urchin egg also provided the first demonstration of the amiloride-sensitive Na$^+$-H$^+$ exchanger, which is an important component of pH regulation in all cells and is a special target for growth factor action in mammalian cells.

Dr. Calzone discussed aspects of the sea urchin system that have been developed for molecular biology approaches. The sea urchin embryo has been used to determine how the genome is differentially transcribed during development. This system has several experimental advantages: a gene transfer system has been accomplished; there is a large collection of cloned genes; there are gene markers for every cell type; and a large quantity of synchronized embryos can be used.

Dr. Calzone has cloned the SM50 gene which is the bimineralization gene in the sea urchin. There are cloned genes for every developmental stage. The cDNA libraries could be a resource to other laboratories. To look for the control regions of the SM50 gene, transgenic animals were produced; however, no general models have been made as yet with transgenic animals because the control regions are very complicated.

Lobster

Studies on neurohormones and neurohormonal systems and how they influence behavior have been carried out with the east coast lobster, *Homarus americanus*. Dr. Kravitz has demonstrated that the posture associated with aggressive behavior in these animals is mimicked when lobsters are injected with the amine, serotonin. By contrast, when injected with the amine octopamine (the lobster equivalent and a phenol analogue of the vertebrate neurotransmitter norepinephrine), animals assume a "submissive-looking" stance. Studies with isolated tissue preparations indicate that these postures result from amine actions on peripheral muscles, which are primed to respond more vigorously, and on the central nervous system where amines cause the readout of opposing motor programs that generate the postures. One of the great advantages of animals like lobster is that the individual neurons involved in behavior can be identified and isolated for physiological, biochemical, and molecular biological studies. Experiments now are centered on the search for identified neurons that may be involved in aggressive behavior in lobsters. Two pairs of serotonin-containing cells with large dendritic and axonal arbors that span much of the lobster central nervous system have been found. These cells act as "gain-setting" elements that enhance the effectiveness of motor programs that lead to postures resembling dominant animals, and that diminish the effectiveness of motor programs triggering a subordinate-like posture.

Barnacles

The muscle cells of the extraordinarily large barnacle, *Balanus nubilis,* that are responsible for depressing the barnacle into its shell, are larger than any others recorded in the animal kingdom. As indicated by Dr. Willows, these muscle cells are 2 mm in diameter and 30 mm long, and so are approximately 100 times larger than their typical mammalian counterparts. These cells may be dissected individually, cannulated to permit alterations of the ionic medium surrounding the contractile proteins, and are conveniently stimulated for hours in controlled perfusion media. These unique experimental features have led to their use in basic muscle research.

Invertebrate diseases

Little is known about invertebrate diseases, and this area of research is important not only in the study of basic biological mechanisms, but also in the maintenance and culturing of these animals. As indicated by Dr. Ralph A. Elston (Battelle), invertebrate disease models to study basic biology are often generated from applied studies in resource and aquaculture management. The requirements of a model are that the disease be transmissible or inducible in the laboratory in a manageable time frame and that hosts both with and free of the disease be made available.

Hemic neoplasia of bivalve molluscs is an example of the use of a mollusc disease to study both basic biological processes and applications to aquaculture management of the disease. Dr. Elston has investigated the basic biological aspects of the etiology and pathogenesis of the disease in the blue mussel *Mytilus edulis.* These studies revealed the progressive fatal nature of the disease, the presence of some individuals which can undergo remission, the transplantable and tranmissable nature of the disease, and the presence of one normal cell cycle that evolves into two apparently anomalous cell cycles. The mussel model now offers some utility to study the basis for remission and means of diagnosing and treating the disease.

Dr. Epel also emphasized that it is critical to understand the pathology of animals such as the sea urchin to successfully culture them. A recent pandemic of the *Diadema* population in the Caribbean resulted in the death of a large portion of these sea urchins due to unknown causes.

Marine Species as Potential Model Systems

Marine invertebrate models for the study of disease processes appear to represent a wide array of potentially fruitful avenues of biomedical research although the field is not well-developed. As stressed by Dr. Elston, further research to develop and apply findings from these models will define their utility.

How does one evaluate a potential marine biomedical model? Dr. Michael J. Greenberg (Whitney Laboratory) suggested four characteristics of a potential model, and illustrated them with (among other systems) the study of the ventral eye of *Limulus* by B-A. Battelle. First, the *process* being investigated must be assessed. If it is a general one, similar in all animals, it is likely to be successfully modelled. The process being investigated with the ventral eye—the mechanisms of modulation of the photoreceptor cells by octopamine—is widely applicable. Second, the *parallelism* between the process in the marine animal and in mammals must be considered. The *Limulus* ventral eye is sufficiently similar to the eyes of vertebrates to be a useful model, but sufficiently different that new discoveries can be made. Third, the *advantages* of the model must be weighed: *e.g.*, the simplicity of the ventral eye and its innervation, and the possibility of making pure photoreceptor cell preparations for biochemical analyses. Also, the location of the circadian clock in the brain rather than the eye (as in vertebrates) is an advantageous difference since the clock can be disconnected from the system and is available as an experimental variable. Fourth, the *animal* itself must be evaluated. In terms of its availability and perishability, the *Limulus* is hardy, easily transported and maintained and can be obtained year-round. Also, the biology of the animal is well-understood.

Crustacea

The crustacean urinary bladder is a potential excellent model system for investigations of renal function. The bladder has a tremendous advantage over other tubular preparations because this tissue is a single cell thick and is large enough to mount in sheets on a conventional flux chamber. Dr. Pritchard has demonstrated that the crustacean urinary bladder shares some characteristics with mammalian proximal tubules: (1) it contains electrically "leaky" epithelium; (2) it resorbs sugars and amino acids; and (3) it secretes organic anions and cations.

The molt cycle, a discontinuous growth strategy of the blue crab, *Callinectes sapidus,* is a useful model for the study of mineral metabolism/regulation under normal conditions. Dr. David Engel (National Marine Fisheries Service, NOAA) collected the hemolymph and digestive glands of the crab at different stages of the molt cycle to determine how molting alters the tissue and cytosolic partitioning of copper and zinc, and the role of metallothionein in these changes. The concentrations of these metals at various stages of the molt indicated that copper and zinc appear to be regulated at the cellular level for the synthesis of metalloproteins, which are necessary for normal growth and survival.

Myxicola

The neuropathy associated with chronic exposure to the solvent n-hexane manifests itself in humans as numbness and burning. n-Hexane is metabolized in the mammalian liver to the potent neurotoxicant, 3,4,-dimethyl-2,5-hexanedione (DMHD). Crosslinking of neurofilaments (NF) is the cause of the neuropathies associated with n-hexane, its metabolites and neurotoxic analogs. Dr. Mary Beth St. Clair (Duke University) used the fanworm, *Myxicola infindibulum,* as an experimental model to study the mechanisms associated with these neuropathies because the large syncytial axon, which is also a source of NF, is so accessible. *Myxicola* is used to screen neurotoxic agents whose putative mechanisms involve covalent crosslinking of NF proteins. Both *in vitro* and *in vivo* studies confirmed that NF are crosslinked in γ-diketone neuropathy. The mechanism of other neurotoxicants, such as CS_2, which produce a neuropathy in mammals identical to the γ-diketone neuropathies, can also be studied.

Tunicates

Dr. Irving Weissman (Stanford University) is using tunicates to investigate the genetics of rejection. *Botryllus schlosseri* has two distinct developmental pathways, sexual and asexual, that lead to indistinguishable adult forms. A single colony of asexually derived, genetically identical blastzooids contain 100 to 1000 individuals. When the growing edges of two colonies come into contact, either fusion or rejection occurs. This allorecognition phenomenon, called colony specificity, is governed by a single highly polymorphic gene locus with multiple codominant alleles, and bears strong resemblance to the vertebrate Major Histocompatibility Complex (MHC). Dr. Weissman has begun to establish, by defined-crosses and self-crosses, Fu/MHC homozygous colonies of the tunicates, and will investigate allorecognition phenomena at the organizational, cellular, and molecular levels.

Elasmobranch fishes

The elasmobranch fishes—*i.e.,* sharks, skates, and rays—provide unique models for elucidating some of the basic developmental and functional mechanisms active in higher vertebrates.

There are three principal modes of fetal or embryonic nutrition in vertebrates: (1) vitellogenesis, or the hepatic preformation of energy rich precursors stored in oocytes prior to ovulation; (2) histotroph, nutrient substances supplied to the embryo in viviparous animals; and (3) placenta, the direct provision of nutrients by a maternal-fetal vascular connection.

Dr. William C. Hamlett (Medical College of Ohio) used the elasmobranch fishes to study the regulation of placentation and the evolution of viviparity. Viviparity in these primitive gnathostomes is of great interest, for within this single group, reproductive processes vary along a continuum from strictly oviparous, reliant exclusively on yolk, to viviparous with a yolk sac placenta. In placental sharks, there is a serial ontogenetic progression in reproductive modes from yolk reliance to histotrophic to placental. The processes of vitellogenesis, histotroph production, and placentation are hormone dependent and interrelated.

Mote Marine Laboratory scientists have used the clearnose skate to examine the effects of environmental agents on reproduction and embryonic development. As described by Dr. Richard Pierce, the skate has great value as a model because of its novel reproductive physiology and behavior. Toxicological effects can be studied at different stages of embryonic development since fertilized females lay eggs over a few months. Thus, sibling embryos covering a broad range of development are available at any time. Since eggs are laid in pairs, one can be maintained as a control at each stage. Also, during the egg laying season, skate ovaries contain ova of vastly differing stages of maturation, ranging from those that are ready to be ovulated, to those that will not mature until the following year. This allows investigation of cumulative uptake of chemical substances at different stages of gamete maturation.

Biomaterials

One consequence of research on marine and freshwater organisms is the discovery of physiologically important molecules. Certain marine natural products that have been recently identified and characterized, have proven to be of enormous biomedical interest and utility.

As explained by Dr. Willows, curiosity about the underlying molecular basis for the bioluminescence of the marine jellyfish *Aequorea* has yielded a purified, and now commercially available, polypeptide whose amino acid sequence has been determined and whose gene has been cloned. Aequorin, the calcium-sensitive protein normally responsible for the natural bioluminescence of certain jellyfish, is widely used in basic research on the actions of ionic calcium in muscle contraction, egg cell fertilization, cell division, and neuronal electrical activity.

Enquiry into the role of unusually high concentrations of peptides found in certain identifiable nerve cells in marine molluscan brains has provided an opportunity to characterize a new family of neuroactive peptides called SCPs, which regulate feeding behavior and heart rate. These peptides are now produced commercially, as are the related monoclonal antibodies useful in tracing their anatomical locations in the brain and actions across the animal kingdom.

Dr. Robert Jacobs (University of California, Santa Barbara) has characterized products from marine species that are natural inhibitors of processes such as the arachidonic acid cascade. For example, manoalide inhibits phospholipase A_2 at the active site of the enzyme, and has anti-inflammatory action. Stypoldione, isolated from algae collected in the South Pacific, causes disorganization of microtubules, most likely by interfering with microtubule assembly. These inhibitors can be used by researchers to elucidate basic mechanisms in a variety of biological processes.

Dr. Christine Benedict (BioPolymers) discussed the applications of a mussel adhesive protein that has been isolated and characterized. This protein attaches to many types of surfaces, both natural and man-made, and withstands a wide range of insult. It has potential application for cell immobilization for tissue, such as bone, teeth, and soft tissue.

Conclusions

As stressed by Dr. Willows, narrowly focused research, sometimes imposed by political or financial pressure, can lead to enormous waste. It is important not to force scientists to tailor their research efforts to fit into a limited number of highly funded popular programs, but to allow a freer pursuit of higher risk research. The rewards that come from pursuing these more creative ideas are frequently the most beneficial and significant.

For example, during the period 1948–1970, if political or funding pressure had driven research on the ionic basis of electrical impulse conduction in nervous systems in the direction of mammalian species, considerable time and money probably would have been wasted. Success in this effort absolutely required the use of the squid giant axon, because of its size and relatively simple combination of sodium and potassium currents, to unravel this mystery. These studies were catalytic to the explosion of progress in medical neurosciences over the succeeding two decades.

Recommendations

We believe that the critical need for biomedical research as related to marine and freshwater organisms is to support the advancement of basic experimental systems. This will require a substantial investment of financial resources, and some risk on the part of federal funding agencies.

Acknowledgments

This conference came into being in a very short time. Only because of the help and cooperation of a number of dedicated individuals were we able to produce a successful program. Unfortunately, due to time constraints, not all of the agencies that fund marine research were represented at the workshop. We did not intentionally exclude any agency, and apologize to those who were not included.

We would like to acknowledge the help of Dr. Joseph Bonaventura, Co-Director of the Marine Biomedical Center at the Duke University Marine Laboratory. His staff, in particular Mrs. Belinda Williford and Mrs. Cindy Baldwin, were highly organized, helpful, and amazingly cheerful and calm throughout the planning of this conference.

We would like to thank Dr. Harlyn O. Halvorson, Director and President of the Marine Biological Laboratory, Woods Hole, who persisted in his efforts to see that this conference materialized. We were sorry that Dr. Halvorson was unable to participate in the conference, but he was certainly there in spirit.

The conference could not have occurred without the financial support of Monsanto Company, in particular Dr. Ernest Jaworski, and North Carolina Biotechnology Center, especially Dr. Charles Hamner. Both of these sponsors were enthusiastic about the scope of the meeting and were very generous in their support.

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